Dual release of peptides and phenolics with antioxidant, ACE-inhibitory and anti-inflammatory activities from pinto bean (*Phaseolus vulgaris* L. var. pinto) proteins by subtilisins

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ABSTRACT

Production of novel functional ingredients from legume proteins is a valuable strategy to boost their food use and intake. Aimed at a better exploitation of the functional properties of pinto bean hydrolysates, the effectiveness of two subtilisins (Alcalase and Savinase) for the dual release of bioactive peptides and phenolics with antioxidant, angiotensin I converting enzyme (ACE) inhibitory and anti-inflammatory activities was investigated. A higher proteolytic efficiency was observed for Alcalase that increased the concentration of small peptides in hydrolysates after 120 min. Savinase improved hydroxycinnamic acids and flavonoids contents in hydrolysates after 90 min due to its higher esterase activity. The most active hydrolysates combined a potent free radical scavenging (326-348 mmol TE/g), ACE inhibitory ($IC_{50}=0.22-0.26$ mg/mL) and anti-inflammatory (28-16% inhibition) activities. Peptide characterization of active hydrolysates confirmed the presence of bioactive fragments derived from phaseolin. These results evidence the promising potential of pinto bean hydrolysates as functional ingredients in the design of healthy foods.

Keywords: pinto bean, proteolysis, bioactive peptides, phenolic compounds, biological activity

Abbreviation

AAE: Ascorbic acid equivalents; ABTS: 2,2'-azinobis 3-ethylbenzothiazoline 6-sulfonate; ACE: Angiotensin I converting enzyme; CAE: Catechin equivalents; CV: cyclic voltammetry; CVD: Cardiovascular diseases; DW: Dry weight; FAE: Ferulic acid equivalents; FRAP: Ferric reducing antioxidant power; HAT: hydrogen atom transfer; ET: Electron transfer; IL-1 β : Interleukin 1 β ; IL-6: Interleukin 6; ORAC: Oxygen radical absorbance capacity; SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis; TE: Trolox equivalents; TP: Total phenolics; TF: Total flavonoids;

1. Introduction

Cardiovascular diseases (CVD) are the biggest cause of global mortality (WHO, 2014). Raised blood pressure (hypertension) is a major cardiovascular risk factor that has been considered as a key target for controlling CVD-related mortality and improving global health. Healthy diets consisting of low sodium and unsaturated fat intake as well as increased consumption of fruits, vegetables and legumes have shown a high impact for reducing the prevalence of hypertension (WHO, 2015).

Nutritionally, legumes are recognized as a good source of proteins, starch, dietary fiber, minerals and vitamins (Vaz Patto et al., 2015). Legume consumption has been associated to the prevention of CVD (Arnoldi, Zanoni, Lammi, & Boschini, 2015; Rebello, Greenway, & Finley, 2014). Positive health effects of legumes on CVD prevention have been ascribed to the presence of a variety of naturally occurring bioactive compounds. Legume proteins are sources of bioactive peptides exhibiting blood pressure-lowering (angiotensin I converting enzyme (ACE)-inhibitory), anti-inflammatory and antioxidant activities once released upon gastrointestinal digestion or enzymatic treatments that may occur during food processing (López-Barrios, Gutiérrez-Urbe, & Serna-Saldívar, 2014). Legumes are also rich in phenolic compounds ranging from 1.1 to 68 mg/g of dry weight (Vaz Patto et al., 2015). Phenolic acids and flavonoids are the most abundant phenolics in legumes and they generally occur as free and bound forms, the latter representing up to 50% of the total phenolic content (Gutiérrez-Urbe, Romo-Lopez, & Serna-Saldívar, 2011; Yeo & Shahidi, 2015). Scientific data evidence the potential of polyphenols to improve cardiovascular health through an array of actions including antioxidant, anti-inflammatory, antihypertensive and antiatherosclerotic activities (Del Rio et al., 2013). Bioactivities and bioavailability of plant polyphenols may be affected by non-covalent and covalent interactions between these compounds and food matrix components (Wang, He, & Chen, 2014). Some processing technologies such as germination, fermentation and enzymatic treatments (Acosta-Estrada, Gutiérrez-Urbe, & Serna-Saldívar, 2014) have been evaluated to avoid these disadvantages increasing soluble to bound phenolics ratio.

The common bean (*Phaseolus vulgaris* L.) is the most important legume species besides soybean in terms of economic value and represents one third of the total world production of pulses (22.5 Mt/year) (FAOSTAT, 2013). Common beans have received increased attention because of their beneficial physiological effects in reducing cardiometabolic risk factors and a broad range of chronic diseases (Hayat, Ahmad, Masud, Ahmed, & Bashir, 2014). Dry beans are the staple food of many countries in America and Asia; however, despite their nutritional value and beneficial effects common beans are largely underexploited and their worldwide consumption is declining (Kohajdová, Karovičová, & Magala, 2013). In this context, one valuable approach to enhance common beans intake and uses is their application in the design of novel and healthy foods.

Aimed at a better exploitation of legumes food products, production of functional hydrolysates containing bioactive peptides from legume seed storage proteins is a topic of great scientific interest (Garcia-Mora, Peñas, Frias, Gomez, & Martinez-Villaluenga, 2015; Luna Vital, González De Mejía, Dia, & Loarca-Piña, 2014; Torruco-Uco, Chel-Guerrero, Martínez-Ayala, Dávila-Ortíz, & Betancur-Ancona, 2009; Valdez-Ortiz, Fuentes-Gutiérrez, Germán-Báez, Gutiérrez-Dorado, & Medina-Godoy, 2012). However, the effect of proteolysis on the release of bound phenolics and how phenolic compounds contribute to the bioactivity of legume hydrolysates have been unexplored. In this study we examine the effectiveness of two proteases from *Bacillus* spp. (also known as subtilisins) to release bioactive peptides and phenolic compounds with antioxidant, ACE inhibitory, and anti-inflammatory activities from pinto bean (*Phaseolus vulgaris* var. pinto) proteins.

2. Materials and methods

2.1. Chemicals and materials

DC protein assay reagents were purchased from Biorad-Laboratories (Hercules, CA, USA). Tripeptide Abz-Gly-Phe(NO₂)-Pro was purchased from Cymit-Quimica (Barcelona, Spain). Dimethyl sulfoxide and 2,2'-azinobis 3-ethylbenzothiazoline 6-sulfonate (ABTS), were purchased from MERK Millipore (Germany). Dulbecco's phosphate buffer saline 10x and reagents for gel

electrophoresis were from Life Technologies (CA, USA). All other reagents were purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise specified.

Pinto beans (*Phaseolus vulgaris* L. var. pinto) were purchased from Semillas Iglesias S. A. (Salamanca, Spain). Seeds were cleaned and stored in polyethylene containers at 4 °C. Commercial food-grade enzymes Alcalase[®] 2.4L FG (2.4 AU/g) and Savinase[®] (16 KNPU/g) were kindly provided by Novozymes (Bagsvaerd, Denmark). The myofibroblast-like cell line CCD-18Co, derived from a human colonic mucosal biopsy, was obtained from the American Type Culture Collection (Rockville, MD, USA).

2.2. Determination of protease and esterase activities in commercial enzymatic preparations

Protease activity of Alcalase and Savinase was determined by a spectrophotometric method using casein as the substrate. Rate of hydrolysis of casein was measured in 50 mM potassium phosphate buffer at 50°C and pH 8 for 10 min. L-tyrosine released from casein was quantified by external calibration (0.05-0.55 µM). Absorbance at 660 nm was measured in a microplate reader (Synergy HT, Biotek, USA). Data were expressed as protease U/mg protein, where units (U) are defined as µmoles of tyrosine released from casein per minute. Esterase activity was determined by a spectrophotometric method using *p*-nitrophenyl butyrate as the substrate as previously described. The rate of hydrolysis of *p*-nitrophenyl butyrate (1 mM) was measured at 50°C in 50 mM sodium phosphate buffer, pH 8. *p*-Nitrophenol was used as standard at a concentration range of 0.12-1 mM to monitor ester hydrolysis colorimetrically. Reactions were followed by measuring the decrease in absorbance at 410 nm in a microplate reader (Synergy HT, Biotek, USA). Enzymatic assays were performed in triplicate. Data were expressed as U/mg protein, where esterase units (U) are defined as µmoles of *p*-nitrophenol released from *p*-nitrophenyl butyrate per minute.

2.3. Preparations of pinto bean protein concentrates and hydrolysates

Seeds were ground using a coffee mill (Moulinex, Allencon, France) and passed through a 60-mesh sieve with 0.5 mm pore size. Protein extraction was performed in water at pH 8 and using flour to solvent ratio of 1:10 (w/v). Flour suspension was stirred at 20 C in an orbital shaker during 1 h (Infors, Switzerland). Flour suspension was centrifuged at 10,000 x g for 15 min, and then, vacuum-filtered using a filter funnel (100-160 m nominal pore size). Finally, filtrates were freeze-dried and stored at -20 C under vacuum in plastic bags.

Freeze-dried pinto bean protein extracts were suspended in deionized water (2%, w/v), equilibrated at 50 C and the pH was adjusted to 8 with 1 M NaOH. Proteolysis was conducted adding Alcalase or Savinase at 0.1 U/mg of soluble protein. Reactions were performed at 50 C, pH 8 for 120 min. Aliquots were withdrawn every 30 min and they were heated at 80 C for 15 min for enzyme inactivation. Hydrolysates were centrifuged at 14,000 x g, at 10 C for 10 min and freeze-dried until further analysis. Two independent replicates were obtained for each enzymatic treatment.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein profile of pinto bean protein concentrate and hydrolysates were analysed by SDS-PAGE in NuPAGE Novex 4-12% Bis-Tris Gels. Electroforesis was run in an XCell-sure lock Mini-Cell at 200 V under non-reducing conditions. The running and sample buffers used were NuPAGE MES-SDS, and NuPAGE LDS, respectively. Gels were stained with SimplyBlue SafeStain for 1 h and destained in deionized water for 2 h. The solution Mark 12TM was used as molecular weight standard.

2.5. Determination of protein and peptide content

Soluble protein concentration of samples was determined in triplicate by the DC Protein Assay using bovine serum albumin as standard at a concentration range from 0.1 to 1 mg/mL. Peptide concentration was measured by the DC Protein Assay in permeates obtained by ultrafiltration of samples through cellulose membranes with 3 kDa molecular weight cut off (Millipore Corporation, Billerica, MA, USA). Results were expressed as mg/g in dry weight (DW).

2.6. Determination of total polyphenol and flavonoid contents

Total phenolic (TP) content was determined in triplicate according to Przygodzka, Zielińska, Ciesarová, Kukurová, & Zieliński (2014). The absorbance was measured at 725 nm in a spectrophotometer (UV-160 1PC, Shimadzu, Japan). TP content was calculated by external calibration using ferulic acid as standard at a concentration range 0.03–1 mg/mL. Results were expressed as mg ferulic acid equivalents (FAE)/g DW. Total flavonoid (TF) content was determined in triplicate according the method described by Zhishen, Mengcheng, & Jianming (1999). The absorbance was measured at 510 nm in a spectrophotometer (UV-160 1PC, Shimadzu, Japan). TF content was calculated by external calibration using (+)-catechin as standard at a concentration range of 0.01–0.2 mg/mL. Results were expressed as mg catechin equivalents (CAE)/g DW.

2.7. Biological activity assays

2.7.1. Antioxidant activity

2.7.1.1. Oxygen Radical Absorbance Capacity (ORAC). ORAC was determined in triplicate as previously reported (Garcia-Mora, Peñas, Frias, & Martínez-Villaluenga, 2014). Fluorescence was measured in a microplate reader (Synergy HT, Biotek, USA) at 485 and 520 nm for emission and excitation wavelengths, respectively. Trolox solutions at a concentration range from 8–160 µM were prepared for external calibration. Results were expressed as mmoles Trolox equivalents (TE)/g DW.

2.7.1.2. Ferric reducing antioxidant power (FRAP). FRAP was evaluated according to Liyana-Pathirana & Shahidi (2007). The absorbance was measured at 700 nm using spectrophotometer (UV-160 1PC, Shimadzu, Japan). Data were obtained by interpolating the absorbance on a calibration curve obtained with ascorbic acid (30–1,000 µM). Results were expressed as µmoles of ascorbic acid equivalents (AAE)/g DW.

2.7.1.3. Scavenging of 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonate) radical cation (ABTS•+) assay. This assay was performed as described by Re et al. (1999). Measurements of absorbance at

734 nm were carried out using the UV-160 temperature-controlled spectrophotometer with a CPS-Controller (Shimadzu, Tokyo, Japan). The standard curve was plotted based on the length of the lag phase versus Trolox concentrations within the range of 0.1–0.75 mM. The antioxidant capacity was expressed as $\mu\text{mol TE/g DW}$.

2.7.1.4. Determination of reducing activity by cyclic voltammetry (CV). A potentiostat/galvanostat (GAMRY, Warminster, PA, USA) was used for voltammetric experiments as it was previously reported (Zielińska, Nagels, & Piskula, 2008). Cyclic voltammetric experiments were performed in ethanol/water (1:1, v/v) and samples (40 mg/mL) mixed with 0.2 M sodium acetate-acetic buffer (80% methanol, pH 5) at a ratio of 1:1 (v/v). The cyclic voltammograms were acquired in the range of -100 to $+1200$ mV at a scanning rate of 100 mV s^{-1} and at 2 mV intervals. Trolox solutions within the concentration range of 0.1–1.5 mM were used and the results were expressed as $\mu\text{mol TE/g DW}$. Triplicate samples were run for each set.

2.7.2. Determination of ACE inhibitory activity

The ACE inhibitory activity was determined in permeates $< 3\text{kDa}$ of bean protein hydrolysates and control samples. Permeates were obtained after ultrafiltration of samples through membranes of 3 kDa pore size (Millipore Corporation, Billerica, MA, USA). Assays were performed in triplicate at a concentration of 1.5 mg/mL as previously described (Garcia-Mora et al., 2014). The IC_{50} values (concentration in mg dry weight/mL that inhibits 50% of the ACE activity) of the most active hydrolysates were calculated by dose response-curves plotted in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).

2.7.3. Inflammatory activity

2.7.3.1. Cell culture maintenance and cell treatment. CCD-18Co colon myofibroblasts were seeded at 6000 cells/cm^2 and were grown in Eagle's minimum essential medium supplemented with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 1.5 g/L sodium

bicarbonate, 100 U mL⁻¹ penicillin, 100 µg mL⁻¹ streptomycin and 10% (v/v) foetal bovine serum at a final pH 7.2–7.4. Cell cultures were maintained at 37° C under a 5% CO₂/95% air atmosphere at constant humidity. Colon myofibroblasts were subcultured when reached >80% of confluence using Trypsin-EDTA (0.25%-0.03%, v/v). Cell cultures between 33 and 34 population doubling levels were used for all experiments.

For cell treatment, cells were cultured in 96-wells plates (BD bioscience, USA) at a concentration of 6000 cells/cm². Cells were grown up to confluence, and were treated with bean hydrolysates at a concentration of 0.01 mg DW/mL. Bean hydrolysates were dissolved in phosphate buffer saline, sonicated and vortexed three times, centrifuged at 10,000 x g for 20 minutes, and filtered (0.22 µm) prior to addition to the culture media. Confluent cells were co-treated with the pro-inflammatory cytokine IL-1β (1 ng/mL) for 24 h. Subsequently, culture medium of the treated cells was collected and stored at -80° C until further analysis.

2.7.3.2. Cell viability assay. Cell viability was estimated using the (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide assay (Cilla, Laparra, Alegria, Barbera, & Farre, 2008). This assay determines *in vitro* cytotoxic effects of treatments described above. Briefly, at the end of the incubation time, optical density was measured at a test wavelength of 570 nm and a reference wavelength of 690 nm using a microplate reader (Synergy H1, Biotek).

2.7.3.3. Determination of interleukin 6 (IL-6). Culture medium was used for the analysis of IL-6 using an ELISA kit from Peprotech (Rocky Hill, NJ, USA). Absorbance was measured in a microplate reader (Infinite M1000 Pro, Tecan) at 450 nm. Results were expressed as ng IL-6/mL. The minimum detection level was 23 pg/mL.

2.8. Identification and quantification of phenolic compounds by reversed phase tandem HPLC-ESI-MS

About 0.1 g of samples were extracted by sonication in 1 mL methanol/water (20/80, v/v) for 30 min at temperature below 40°C. A second sample extraction was performed in 100% methanol as described above. After extraction samples were centrifuged at 16,100 x g and 4 °C (Centrifuge 5415R, Eppendorf, Germany) for 5 min and stored in a capped vial at 4°C until further analysis.

Aliquots of extracts (2 µL) were injected into a LC system (LC-200, Eksigent, USA) consisted of a dual-channel pump, column oven, autosampler (set at 4°C) and system controller coupled with mass spectrometer (QTRAP 5500, AB SCIEX, Canada) consisting of a triple quadrupole, ion trap and ion source of electro-spray ionization (ESI) and controlled by the Analyst 1.5.1. Chromatographic separation was performed at 45 °C at a flow rate of 15 µL/min on HALO C₁₈, 2.7 µm, 0.5 x 50 mm column (Eksigent, USA). Compounds were eluted in a gradient system composed of water/formic acid (99.05/0.95, v/v, phase A) and acetonitrile/formic acid (99.05/0.95, v/v, phase B). Gradient was composed: 5-5-90-90-5-5% B in gradient of time 0-0.1-2-2.5-2.7-3 min. Qualitative and quantitative analysis was made using Multiple Reaction Monitoring (MRM) method for appropriate external standards (Supplementary Table 1). The calibration curves (the range of 1-100 nM) was linear with a correlation coefficient of 0.97. Optimal MS/MS ESI conditions of analysis are presented in Supplementary Table 2.

2.9. Identification of bioactive peptides by tandem nanoUPLC-ESI-MS

For peptide identification, 10 µg of the most active hydrolysates were desalted using a reverse phase (PorosR2[®]) chromatography with 80% acetonitrile/0.1% trifluoroacetic acid. Later, the peptides were dried and resuspended with 10 µL 0.1% TFA. An aliquot of 5 µl of resuspended sample were injected in the linear trap quadrupole Orbitrap Velos (Thermo Scientific). Peptides were loaded onto a C18-A1 ASY-Column 2 cm precolumn (Thermo Scientific) and then eluted onto a Biosphere C18 column (inner diameter 75 µm, 15 cm long, 3 µm particle size) (NanoSeparations) at a flow rate of 250 nL/min on a nanoEasy high-performance liquid chromatography (Proxeon) coupled to a nanoelectrospray ion source (Proxeon).

The mobile phases used consisted of 0.1% formic acid/2% acetonitrile (solvent A) and 0.1% formic acid in 100% acetonitrile (solvent B). The solvent gradient was from 100% A to 35% B in 140 min. Mass spectra were acquired in the positive ion mode. Full-scan MS spectra (m/z 400-1800) were acquired with a target value of 1,000,000 at a resolution of 30,000 at m/z 400 and the 15 most intense ions were selected for collision induced dissociation fragmentation in the linear trap quadrupole with a target value of 10,000 and normalized collision energy of 38%. Precursor ion charge state screening and monoisotopic precursor selection were enabled. Singly charged ions and unassigned charge states were rejected. Dynamic exclusion was enabled with a repeat count of 1 and exclusion duration of 30s.

Proteome Discoverer 1.4.288 (Thermo) with MASCOT 2.3 was used to search in NCBI database, taxonomy Viridiplantae (1530236 sequences sequences) and a home-made database with the *Phaseolus vulgaris* entries in UniProt. Database search parameters used were the following: peptide tolerance, 10 ppm; fragment ion tolerance, 0.8 Da; none enzyme and variable modification, methionine oxidation. In all peptide identification, the probability scores were greater than the score fixed by Mascot as significant with a p -value < 0.05 . The automatic decoy database search function of Protein Discover was enabled to allow estimation of false discovery rate (FDR). Sequence identification with reported biological activity was compared with bioactive peptides sequences submitted to the BIOPEP database (<http://www.uwm.edu.pl/biochemia/index.php/pl/biopep>).

2.10. Statistical analysis

Results are presented as mean values $\pm$ standard deviation. Data were subjected to one-way analysis of variance (ANOVA) by Statgraphics Centurion XVI software, version 16.1.17 (Statistical Graphics Corporation, Rockville, Md). Differences between samples were compared by using a Duncan's multiple-range test at $p \leq 0.05$ probability levels.

3. Results

3.1. Proteolytic pattern of hydrolysis

Figure 1 shows the electrophoretic protein profile under denaturing and non-reducing conditions of the pinto bean protein concentrate (control) and their respective hydrolysates obtained at different times (30-120 min). Protein concentrate (lane C(-)) showed intense bands with molecular weights between 97 to 10 kDa. Bands corresponding to phaseolin (25, 45 and 50 kDa) and phytohemagglutinins, α -amilase inhibitor and α -amylase β subunit (32 and 18, 15 kDa, respectively) were readily digested to smaller fractions (< 15 kDa) within 30 min of Alcalase treatment. Complete degradation of these proteins was observed after 120 min of Alcalase digestion. The intensity of hydrolysis products was decreasing gradually with time suggesting a further degradation of peptides into much smaller fractions. Savinase treatment also resulted in a considerable degradation of the main protein bands yielding smaller protein fragments (< 20 kDa) within 30 min. Further hydrolysis times showed little changes in the protein profile. Some phaseolin subunits and phytohemagglutinin protein bands remained stable after 120 min and only a slight decrease in the intensity of bands < 20 kDa was observed, indicating the generation of much smaller peptides. Nevertheless, as can be observed from the figure, the proteolytic efficiency of Savinase was lower than that of Alcalase.

3.2. Concentration of peptides < 3 kDa, total phenolics and flavonoids in pinto bean hydrolysates

It is well-known that bioactive peptides are short amino acid sequences (Udenigwe & Aluko, 2012). To evaluate the cost-effectiveness of pinto bean proteolysis by subtilisins to release small peptides, peptide concentration was measured in 3 kDa permeates from hydrolysates obtained at different times of hydrolysis (Table 1). Concentration of peptides < 3 kDa increased time-dependently by both enzymatic treatments ($P<0.05$) at a similar rate than that observed for proteolysis in SDS-PAGE. The highest peptide concentrations were reached after 120 min of hydrolysis and, Alcalase yielded higher values (544 mg/g) than Savinase (508 mg/g) ($P<0.05$).

The effect of pinto bean proteolysis by subtilisins at different times of hydrolysis on total phenolic and flavonoid content is presented in Table 1. Alcalase and Savinase proteolysis markedly increased total phenolic content of hydrolysates in a time-dependent manner ($P<0.05$). The highest total phenolic content was observed in hydrolysates obtained by Alcalase after 120 min (12.7 mg FAE/g) and Savinase after 90 min (12.9 mg FAE/g), with no significant differences among them ($P>0.05$). Surprisingly, Alcalase treatment did not affect total flavonoid concentration ($P>0.05$) whereas Savinase digestion for 30 min significantly increased total flavonoid concentration (4.1 mg CAE/g) ($P<0.05$). Longer hydrolysis times yielded a similar flavonoid content with the exception of Savinase treatments for 120 min resulting in values non significantly different from control ($P>0.05$).

3.3. Antioxidant activity of pinto bean protein hydrolysates

Table 2 shows the effect of two subtilisin treatments and hydrolysis times on antioxidant activity of pinto bean hydrolysates. The antioxidant activity was determined by four methods ORAC, FRAP, ABTS, and CV based on different antioxidant mechanisms. ORAC assay is based on hydrogen atom transfer (HAT) mechanism, FRAP and CV assays use electron transfer (ET) mechanisms and ABTS method comprise of mixed HAT and ET mechanisms (Craft, Kerrihard, Amarowicz, & Pegg, 2012). Alcalase and Savinase treatments considerably increased the antioxidant activity, as determined by ORAC and ABTS assay, compared to control ($P<0.05$). The highest ORAC values were obtained after 120 min for both enzymes (348.99 mmol TE/g for Alcalase and 326.11 mmol TE/g for Savinase), with no significant differences among them ($P > 0.05$). ABTS radical scavenging activity increased progressively and reached a maximum after 120 min (120 μ mol TE/g) as consequence of Alcalase proteolysis whilst it reached a plateau after 30 min in the Savinase treatment (123 μ mol TE/g) ($P>0.05$). Regarding FRAP, enzymatic treatments generally brought about a significant reduction in the electron donor ability of hydrolysates, with the exception of Alcalase proteolysis for 60 min that resulted in a slight increase ($P<0.05$). Finally, both enzymes improved the electro

reducing power of hydrolysates, as measured by CV, although this effect was dependent of hydrolysis time. Shorter hydrolysis times (30 min) gave rise the highest electro reducing power in hydrolysates obtained by Alcalase (26.0 $\mu\text{mol TE/g}$) whereas longer digestion times (90 and 120 min) were needed to increase this activity in Savinase hydrolysates (24.1-25.3 $\mu\text{mol TE/g}$). Significant positive correlations were found between antioxidant activity (measured as ORAC, ABTS and CV) and total phenolic/peptide content of Alcalase and Savinase hydrolysates ($P<0.05$). In contrast, FRAP was negatively correlated with peptide and total phenolic contents ($P<0.05$).

3.4. ACE inhibitory activity of pinto bean hydrolysates

ACE inhibitory activities were determined in 3 kDa permeates of samples hydrolysed by Alcalase and Savinase at different times (Figure 2A). Captopril at 0.1 μM was used as positive control (C+) displaying a 98% ACE inhibition. As compared to positive control, 3 kDa permeates of non-hydrolysed samples (0 min) displayed a weak ACE inhibition (21%) at a concentration of 1.5 mg DW/mL. Enzymatic treatments brought about a significant improvement in the ACE inhibitory activity of the 3 kDa fraction ($P<0.05$). Alcalase hydrolysis gradually increased ACE inhibitory activity up to 62% inhibition within 60 min and no improvements were observed upon further digestion ($P>0.05$). Savinase treatment for 90 min increased ACE inhibitory activity of hydrolysates time-dependently whereas longer hydrolysis times led to a decrease of ACE inhibition ($P<0.05$). Most active hydrolysates produced by Alcalase for 120 min and Savinase for 90 min exhibited IC_{50} values of 0.26 and 0.23 mg /mL, respectively, with no significant differences among them ($P<0.05$). Significant positive correlations ($P<0.05$) were found between ACE inhibitory activity vs. peptides or total phenolics content of Alcalase ($r = 0.81$ and $r = 0.85$) and Savinase ($r = 0.86$ and $r = 0.78$) hydrolysates. These findings suggest that either peptides < 3 kDa or phenolic compounds are contributing to ACE inhibitory activity of bean hydrolysates.

3.5. Anti-inflammatory activity of pinto bean hydrolysates

Figure 2B presents the effect of Alcalase and Savinase treatment at different times on anti-inflammatory activity of hydrolysates. Cell treatments with pinto bean hydrolysates at a concentration of 0.01 mg/mL did not affect CCD-18Co cells viability (data not shown). Unstimulated cells with IL-1 β (C-) secreted negligible IL-6 amounts as compared to IL-1 β stimulated cells (C+) that produced 105.51 ng of IL-6 /mL. Cell treatments with hydrolysates (0.01 μ g/mL) obtained by Alcalase at 15, 30 and 120 min and Savinase at 60 and 90 min resulted in a statistically significant reduction in the secretion of IL-6 by IL-1 β stimulated CCD-18Co cells (P<0.05). The hydrolysate obtained by Alcalase at 120 min was the one that most potently inhibited the IL-6 secretion by IL-1 β stimulated CCD-18Co cells (28% inhibition vs. control) followed by hydrolysates produced by Alcalase for 15 min and Savinase for 60 or 90 min (16% inhibition vs. control).

3.6. Phenolic and peptide profile of the most active pinto bean hydrolysates produced by Alcalase and Savinase

Bean hydrolysates obtained by Alcalase treatment for 120 min and Savinase for 90 min were selected for peptides and phenolics identification on the basis of its higher multifunctional properties including antioxidant, ACE inhibitory and anti-inflammatory activities.

3.6.1. Peptide profile

Table 3 shows the list of the most abundant peptides identified in selected pinto bean hydrolysates. These peptides were fragments from α -type phaseolin precursor (accession n. 21036), phaseolin (accession n. 403594) and β -type phaseolin storage protein (accession n. 169323). Peptides derived from the latter protein were only found in Savinase hydrolysates. This fact joined to the presence of unique amino acid sequences in both hydrolysates indicates that Alcalase and Savinase have different enzyme specificities. Using BIOPEP tools and literature data (Oseguera-Toledo, De Mejia, Dia, & Amaya-Llano, 2011), we found that partial structure of most of these peptides possessed one

or a combination of bioactivities including antioxidant (underlined sequences), ACE inhibitory (bolded amino acid sequence), and anti-inflammatory (highlighted sequences) activities.

3.6.2. Phenolic profile

Table 4 shows the individual and total phenolic content of pinto bean protein concentrate (control) and selected hydrolysates. Hydroxycinnamic compounds were detected as free acids in pinto bean protein concentrates and accounted for 20% of the total phenolic content, being ferulic acid the major compound. Sinapic and *p*-coumaric acids were also identified as minor compounds in this non-flavonoid group. Flavan-3-ols were the most abundant phenolics in pinto bean protein concentrates accounting for approximately 44.4% of the total phenolic content. Catechin was the major flavanol (41.24 µg/g) followed by epicatechin (15.23 µg/g). Flavonols were the less abundant family of phenolics (12% of total phenolic content) in protein concentrates. In this group quercetin glycosides were the predominant compounds although quercetin aglycone and isorhamnetine, myricetin and kaempferol glycosides were also detected. Anthocyanins represented 20% of the total phenolic content in protein concentrates being found mainly as pelargonidin glycosides. Cyanidin aglycone and glycosides as well as malvidine glycosides were also identified as minor compounds of this phenolic family.

Phenolic profile of pinto bean hydrolysates obtained by Alcalase for 120 min showed slight differences compared to protein concentrate (control). Total phenolic content (121.22 µg/mL) was found similar to control ($P>0.05$). A little decrease was observed in the concentration of epicatechin and quercetin 3-malonylglucoside whereas cyanidin and malvidin 3-glucosides content were higher than control ($P<0.05$). Interestingly, significant changes were detected in the phenolic profile of hydrolysates produced by Savinase for 90 min resulting in an approximately 2-fold increase of the total phenolic content. Savinase proteolysis led to small reduction of epicatechin, quercetin 3-malonylglucoside and pelargonidin 3-malonylglucoside concentrations ($P<0.05$). However, it is worth to note that this enzymatic treatment gave rise to 1.8- and 3-fold increase of catechin and

pelargonidin concentrations, respectively. Moreover, less significant increases of minor compounds such as ferulic acid, quercetin 3-xylosylglucoside and malvidin 3-glucoside were found. To explain the higher efficiency of Savinase to increase phenolic content in hydrolysates, esterase activity was measured in both enzymatic preparations. Results showed that esterase activity of Savinase was 3 times higher (1.00 ± 0.04 U/mg protein) than Alcalase (0.33 ± 0.05 U/mg protein) in the assayed conditions ($P < 0.05$) in consistency with differences observed in the phenolic concentrations of hydrolysates.

4. Discussion

Health organizations recommend pulse consumption as part of a healthy diet due to vast evidence of their protective and therapeutic effects on chronic diseases (Rebello et al., 2014). As consequence, initiatives have been developed to improve their production, intake and uses (America Pulse Association, 2010). In this context, the use of legumes as functional ingredients for the development of healthy foods are being explored (López-Barrios et al., 2014). Different legume proteins are being studied for their potential to generate bioactive peptides, although a careful choice of a suitable enzyme and the control of hydrolysis time, are crucial for obtaining desirable bioactive amino acid sequences and functional properties. The raw material for production of functional legume hydrolysates are protein concentrates ($< 80\%$ protein) or protein isolates ($> 90\%$) (López-Barrios et al., 2014). During preparation of legume protein concentrates/isolates, soluble phenolic compounds may be extracted (Martínez-Villaluenga et al., 2009) and can interact non-covalently with storage proteins. On the other hand, during water alkaline extraction of proteins soluble fiber components can be also isolated (Boye et al., 2010) and, as consequence, bound phenolics might be present in the resulting product. Despite the extensive research on functional legume hydrolysates, none of the studies have focused on the effect of enzymatic hydrolysis on the release of bound phenolics and how this fact affect to soluble phenolic concentration and bioactivity of hydrolysates. This study shows for the first time the effectiveness of food-grade commercial proteases from *Bacillus* spp. to

produce hydrolysates with bioactive peptides and phenolic compounds that result in a desirable combination of antioxidant, ACE inhibitory and anti-inflammatory activities.

The main storage protein of common bean (*Phaseolus vulgaris* L.) is phaseolin, a heterogeneous 7S globulin composed of 2 to 6 subunits that constitutes up to 50% of total seed protein (Montoya, Lallès, Beebe, & Leterme, 2010). Phaseolin is highly resistant to proteolysis due to its compact and rigid structure rich in β -sheet domains, a high degree of glycosylation and high hydrophobicity. Our results indicate that phaseolin was susceptible to Alcalase and Savinase action probably due to their broad specificity. Susceptibility of phaseolin to Alcalase and other proteases has also been reported by other authors (Oseguera-Toledo et al., 2011; Rui, Boye, Simpson, & Prasher, 2012). Nevertheless, there is a clear variability in the proteolytic rate of phaseolin by Alcalase among different studies. This variability may be ascribed to a different enzyme to substrate ratio or the structural heterogeneity of phaseolin among common bean cultivars (Montoya et al., 2008). Phaseolin has a high source of variation in the precursor subunits profiles ($\alpha\beta$, $\alpha\beta\beta$ and $\beta\beta$ for phaseolins type S, T and I, respectively), in the carbohydrate composition and number of phosphate and carbohydrate binding sites (Montoya et al., 2010). As compared to Alcalase, Savinase exhibited a lower proteolytic efficiency which may be attributed to differences in enzyme specificity. These results are in contrast with our previous findings showing that Savinase was the most efficient enzyme to hydrolyse lentil globulins (Garcia-Mora et al., 2014).

Alcalase and Savinase treatments resulted in a gradual increase of small peptides (< 3 kDa) in a time-dependent manner although Alcalase gave rise to a higher accumulation of peptides than Savinase, in consistency with the differences observed in their proteolytic efficiency. Alcalase have been proven to be effective for releasing small peptides from many other legumes such as chickpea (Medina-Godoy et al., 2012), lima and jamapa beans (Torruco-Uco et al., 2009) and lentil (Barbana & Boye, 2011). However, Savinase has been less used in food technology although it has recently shown a

good potential to produce small peptides from lentil proteins (Garcia-Mora et al., 2015; Garcia-Mora et al., 2014).

The present study has demonstrated that Alcalase and Savinase treatments increase the concentration of total phenolics in a time-dependent manner. Interestingly, the comparative assessment of the phenolic composition of hydrolysates produced by Alcalase at 120 min and Savinase at 90 min (Table 4) indicated that the latter treatment lead to a 2-times higher content of total phenolics as consequence of a marked increased in hydroxycinnamic acids (ferulic acid), flavan-3-ols (catechin) and anthocyanins (pelargonidin-3-glucoside) families. Our results are consistent with a recent investigation showing the release of flavonoids (apigenin-C glycosides) bound by ionic bridges and hydrogen bonding to γ -conglutin from lupin (Czubinski et al., 2012). Higher concentration of hydroxycinnamic acids observed in Savinase hydrolysates obtained after 90 min can be also ascribed to the higher esterase activity found in the Savinase enzymatic preparation. Feruloyl esterases are involved in the de-esterification of dietary fiber, releasing hydroxycinnamates and derivatives (Acosta-Estrada et al., 2014). These enzymes have been described in different microorganisms including *Bacillus* spp. (Donaghy, Kelly, & McKay, 1998), bacteria from which Alcalase and Savinase preparations were obtained.

Pinto bean hydrolysates produced by Alcalase and Savinase displayed a combination of antioxidant, ACE-inhibitory and anti-inflammatory activities, therefore, their potential as functional food ingredients should be encouraged. Amelioration of oxidative stress through dietary antioxidants is considered a promising strategy in the prevention of CVD (Leopold, 2015). In general, pinto bean proteolysis by Alcalase and Savinase gradually increased radical scavenging activity with hydrolysis time mainly by mechanisms of hydrogen donation. As indicated by correlation analysis, this effect on radical scavenging activity (measured by ORAC and ABTS methods) could be attributed to a higher accumulation of small peptides and phenolic compounds during hydrolysis. In agreement with our results, previous studies have reported that Alcalase hydrolysis of other protein sources provides

smaller peptide fractions with a better radical scavenging activity than high molecular weight fractions (Samaranayaka & Li-Chan, 2011). The effectiveness of Alcalase to produce antioxidant peptides has been related to its broad specificity and its preferential breakdown of hydrophobic amino acids (Gupta, Beg, & Lorenz, 2002) that act as hydrogen donors (Samaranayaka & Li-Chan, 2011). In general, iron reducing activity diminished as consequence of both subtilisin treatments, effect that was negatively correlated with higher contents of small peptides and total phenolics. In agreement with our results, other authors observed that higher reducing power in hemp hydrolysates content is linked to a larger peptide size (Girgih, Udenigwe, & Aluko, 2011). Pinto bean hydrolysates also displayed a weak electro reducing ability as mechanism of antioxidant activity that was slightly improved with proteolytic treatments at certain hydrolysis times.

ACE is a key enzyme in the renin-angiotensin aldosterone system involved in the regulation of blood pressure (Bernstein et al., 2013). ACE inhibition results in a vasodilatation through a reduction of angiotensin II levels. Clinical studies have demonstrated that ACE inhibitors significantly reduce the morbidity and mortality of patients with myocardial infarction or heart failure (Guerrero et al., 2012). Therefore, consumption of foods containing ACE inhibitors in a daily basis could be considered a promising strategy in the prevention or management of CVD. In this study, ACE inhibitory activity was improved gradually with hydrolysis time in agreement with previous studies (Medina-Godoy et al., 2012; Rui et al., 2012), being this effect related to a higher content of peptides < 3 kDa and phenolic compounds as indicated by correlation analyses. Smaller peptides fractions have been found more effectively inhibiting ACE (Wan Mohtar, Hamid, Abd-Aziz, Muhamad, & Saari, 2013) probably due to the fact that the active site blocks the access to large polypeptides (Norris and Fitzgerald, 2013). The IC₅₀ values of the most active pinto bean hydrolysates was within the range of reported values for ACE inhibitory hydrolysates from other legumes (0.06-0.77mg/g) (Garcia-Mora et al., 2015; Garcia-Mora et al., 2014; López-Barrios et al., 2014) and food protein sources (Hernández-Ledesma, Del Mar Contreras, & Recio, 2011).

Inflammation is part of the host defense mechanisms against external stimuli that causes injuries. However, uncontrolled and aberrant inflammatory response has been often considered as one of the underlying pathophysiological mechanisms of hypertension and atherosclerosis (Montecucco, Mach, & Pende, 2013). Therefore, control of excessive inflammation through diet is important to maintain health and wellness. Human colon cells (CCD-18Co) treated by IL-1 β were used as a cell model of inflammation in the present study to evaluate the anti-inflammatory activity of pinto bean hydrolysates. IL-1 β is a pro-inflammatory cytokine produced *in vivo* by activated macrophages and induces the secretion of IL-6 by both immune and non-immune cells in response to infection and injury (Dinarello, 2009). Pinto bean hydrolysates reduced significantly IL-6 secretion by the IL-1 β stimulated human colon cells, although this effect was dependent on the enzyme type and hydrolysis time. The hydrolysate obtained by Alcalase after 120 min was the most potent reducing IL-6 secretion by the IL-1 β -stimulated myofibroblasts. In agreement with our findings, anti-inflammatory activity of hydrolysates from common bean varieties Pinto Durango and Negro 8025 produced by Alcalase and pepsin-pancreatin has also been described in other cell models of inflammation such as lipopolysaccharide activated macrophages RAW267.4 (Oseguera-Toledo et al., 2011). These authors suggested as mechanisms of anti-inflammatory activity exerted by the hydrolysates the inhibition of the activation of the transcription factor NF- κ B and the downregulation of enzymes gene expression such as inducible nitric oxide synthase and cyclooxygenase-2 involved in the synthesis of pro-inflammatory molecules.

The multiple bioactivities displayed by pinto bean hydrolysates can be explained by the enzymatic release of bioactive peptides and phenolic compounds. This was confirmed by an exhaustive analysis of the peptide and phenolic fractions of the most active hydrolysates. Results indicated that most abundant peptides in pinto bean hydrolysates contained reported antioxidant, ACE inhibitory and anti-inflammatory amino acid sequences in part of their primary structure. Moreover, structural features at the C-terminal tripeptide for the blockage of ACE active site (Norris and Fitzgerald, 2013)

were fulfilled by most of the peptides identified in bioactive hydrolysates. These structural features for ACE inhibition included the presence of bulky hydrophobic residues, aromatic or branched side chains, proline (P) at 1 or more positions, positively charged Arg (R) and Lys (K) residues at position 2, Tyr (T), Phe (F), and Trp (W) residues at position 3. Some identified peptides in pinto bean hydrolysates (for instance VNPDPKEDLRRIQL, VVNEGAHVE, VVNEGAHVELVGPK) also showed molecular characteristics described for antioxidant activity such as Val (V) at the N-terminus of the peptides, and Pro (P), His (H), in their sequences (Samaranayaka & Li-Chan, 2011). Phenolic compounds identified in hydrolysates may be also contributors to the bioactivity of hydrolysates. Structure activity studies have shown that *p*-coumaric and ferulic acid have a stronger antioxidant activity (free radical-scavenging, transition metal-chelating, and singlet oxygen quenching capacity) than other cinnamic and hydroxibenzoic acid derivatives. Catechin and epicatechin have been found among the most potent flavonoids inhibiting ACE activity *in vitro* (Guerrero et al., 2012). On the other hand, in a recent study quercetin, kaempferol and myricetin provided a greater modulation of inflammation in endothelial cell cultures compared with hydroxycinnamic acids such as *p*-coumaric, caffeic and caftaric acids (Calabriso et al., 2015). All these phenolic compounds mentioned above were detected in selected pinto bean hydrolysates.

5. Conclusions

This study demonstrates that there is a benefit in the application of subtilisins to release bioactive peptides and phenolic compounds from pinto bean proteins with potential antioxidant, ACE inhibitory and anti-inflammatory activities. Enzyme type and hydrolysis time were important factors affecting the bioactive composition of hydrolysates. Pinto bean hydrolysates obtained by Alcalase after 120 min were characterized by a higher concentration of small peptides < 3kDa. Interestingly, this is the first study showing an outstanding esterase activity of Savinase responsible for a 2-fold increase in the total phenolic concentration of the most active hydrolysates. Therefore, hydrolysates produced by Savinase after 90 min were composed of a markedly higher concentration

of ferulic acid, catechin and pelargonidin-3-glucoside than Alcalase hydrolysates. The identification of the most abundant peptides in the most active hydrolysates support the potential of both enzymatic treatments to efficiently release bioactive peptides from pinto bean proteins.

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REFERENCES

- Acosta-Estrada, B. A., Gutiérrez-Urbe, J. A., & Serna-Saldívar, S. O. (2014). Bound phenolics in foods, a review. *Food Chemistry*, 152, 46-55. doi: 10.1016/j.foodchem.2013.11.093
- Arnoldi, A., Zanoni, C., Lammi, C., & Boschini, G. (2015). The Role of Grain Legumes in the Prevention of Hypercholesterolemia and Hypertension. *Critical Reviews in Plant Sciences*, 34, 144-168. doi: 10.1080/07352689.2014.897908
- Barbana, C., & Boye, J. I. (2011). Angiotensin I-converting enzyme inhibitory properties of lentil protein hydrolysates: Determination of the kinetics of inhibition. *Food Chemistry*, 127(1), 94-101. doi: 10.1016/j.foodchem.2010.12.093
- Bernstein, K. E., Ong, F. S., Blackwell, W. L. B., Shah, K. H., Giani, J. F., Gonzalez-Villalobos, R. A., . . . Fuchs, S. (2013). A modern understanding of the traditional and nontraditional biological functions of angiotensin-converting enzyme. *Pharmacological Reviews*, 65(1), 1-46. doi: 10.1124/pr.112.006809
- Boye, J. I., Aksay, S., Roufik, S., Ribéreau, S., Mondor, M., Farnworth, E., & Rajamohamed, S. H. (2010). Comparison of the functional properties of pea, chickpea and lentil protein concentrates processed using ultrafiltration and isoelectric precipitation techniques. *Food Research International*, 43(2), 537-546. doi: 10.1016/j.foodres.2009.07.021

- Calabriso, N., Scoditti, E., Massaro, M., Pellegrino, M., Storelli, C., Ingrosso, I., . . . Carluccio, M. A. (2015). Multiple anti-inflammatory and anti-atherosclerotic properties of red wine polyphenolic extracts: differential role of hydroxycinnamic acids, flavonols and stilbenes on endothelial inflammatory gene expression. *European Journal of Nutrition*. doi: 10.1007/s00394-015-0865-6
- Cilla, A., Laparra, J. M., Alegria, A., Barbera, R., & Farre, R. (2008). Antioxidant effect derived from bioaccessible fractions of fruit beverages against H₂O₂-induced oxidative stress in Caco-2 cells. *Food Chemistry*, 106(3), 1180-1187. doi: 10.1016/j.foodchem.2007.07.059
- Craft, B. D., Kerrihard, A. L., Amarowicz, R., & Pegg, R. B. (2012). Phenol-Based Antioxidants and the In Vitro Methods Used for Their Assessment. *Comprehensive Reviews in Food Science and Food Safety*, 11(2), 148-173. doi: 10.1111/j.1541-4337.2011.00173.x
- Czubinski, J., Dwiecki, K., Siger, A., Kachlicki, P., Neunert, G., Lampart-Szczapa, E., & Nogala-Kalucka, M. (2012). Release of flavonoids from lupin globulin proteins during digestion in a model system. *Journal of Agricultural and Food Chemistry*, 60(7), 1830-1836. doi: 10.1021/jf2042592
- Del Rio, D., Rodriguez-Mateos, A., Spencer, J. P. E., Tognolini, M., Borges, G., & Crozier, A. (2013). Dietary (poly)phenolics in human health: Structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxidants and Redox Signaling*, 18(14), 1818-1892. doi: 10.1089/ars.2012.4581
- Dinarello, C. A. (2009) Immunological and inflammatory functions of the interleukin-1 family. *Vol. 27. Annual Review of Immunology* (pp. 519-550).
- Donaghy, J., Kelly, P. F., & McKay, A. M. (1998). Detection of ferulic acid esterase production by *Bacillus* spp. and lactobacilli. *Applied Microbiology and Biotechnology*, 50(2), 257-260. doi: 10.1007/s002530051286
- Garcia-Mora, P., Peñas, E., Frias, J., Gomez, R., & Martinez-Villaluenga, C. (2015). High-pressure improves enzymatic proteolysis and the release of peptides with angiotensin i converting enzyme inhibitory and antioxidant activities from lentil proteins. *Food Chemistry*, 171, 224-232. doi: 10.1016/j.foodchem.2014.08.116

- Garcia-Mora, P., Peñas, E., Frias, J., & Martínez-Villaluenga, C. (2014). Savinase, the most suitable enzyme for releasing peptides from lentil (*Lens culinaris* var. Castellana) protein concentrates with multifunctional properties. *Journal of Agricultural and Food Chemistry*, 62(18), 4166-4174.
- Girgih, A. T., Udenigwe, C. C., & Aluko, R. E. (2011). In vitro antioxidant properties of hemp seed (*Cannabis sativa* L.) protein hydrolysate fractions. *JAOCs, Journal of the American Oil Chemists' Society*, 88(3), 381-389. doi: 10.1007/s11746-010-1686-7
- Guerrero, L., Castillo, J., Quiñones, M., Garcia-Vallvé, S., Arola, L., Pujadas, G., & Muguerza, B. (2012). Inhibition of Angiotensin-Converting Enzyme Activity by Flavonoids: Structure-Activity Relationship Studies. *PLoS ONE*, 7(11). doi: 10.1371/journal.pone.0049493
- Gupta, R., Beg, Q., & Lorenz, P. (2002). Bacterial alkaline proteases: Molecular approaches and industrial applications. *Applied Microbiology and Biotechnology*, 59(1), 15-32. doi: 10.1007/s00253-002-0975-y
- Gutiérrez-Urbe, J. A., Romo-Lopez, I., & Serna-Saldívar, S. O. (2011). Phenolic composition and mammary cancer cell inhibition of extracts of whole cowpeas (*Vigna unguiculata*) and its anatomical parts. *Journal of Functional Foods*, 3(4), 290-297. doi: 10.1016/j.jff.2011.05.004
- Hayat, I., Ahmad, A., Masud, T., Ahmed, A., & Bashir, S. (2014). Nutritional and Health Perspectives of Beans (*Phaseolus vulgaris* L.): An Overview. *Critical Reviews in Food Science and Nutrition*, 54(5), 580-592. doi: 10.1080/10408398.2011.596639
- Hernández-Ledesma, B., Del Mar Contreras, M., & Recio, I. (2011). Antihypertensive peptides: Production, bioavailability and incorporation into foods. *Advances in Colloid and Interface Science*, 165(1), 23-35. doi: 10.1016/j.cis.2010.11.001
- Kohajdová, Z., Karovičová, J., & Magala, M. (2013). Effect of lentil and bean flours on rheological and baking properties of wheat dough. *Chemical Papers*, 67(4), 398-407. doi: 10.2478/s11696-012-0295-3
- Leopold, J. A. (2015). Antioxidants and coronary artery disease: From pathophysiology to preventive therapy. *Coronary Artery Disease*, 26(2), 176-183. doi: 10.1097/MCA.0000000000000187
- Liyana-Pathirana, C. M., & Shahidi, F. (2007). The antioxidant potential of milling fractions from breadwheat and durum. *Journal of Cereal Science*, 45(3), 238-247. doi: 10.1016/j.jcs.2006.08.007

- López-Barrios, L., Gutiérrez-Urbe, J. A., & Serna-Saldívar, S. O. (2014). Bioactive Peptides and Hydrolysates from Pulses and Their Potential Use as Functional Ingredients. *Journal of Food Science*, 79(3), R273-R283. doi: 10.1111/1750-3841.12365
- Luna Vital, D. A., González De Mejía, E., Dia, V. P., & Loarca-Piña, G. (2014). Peptides in common bean fractions inhibit human colorectal cancer cells. *Food Chemistry*, 157, 347-355. doi: 10.1016/j.foodchem.2014.02.050
- Martínez-Villaluenga, C., Zieliński, H., Frias, J., Piskula, M. K., Kozłowska, H., & Vidal-Valverde, C. (2009). Antioxidant capacity and polyphenolic content of high-protein lupin products. *Food Chemistry*, 112(1), 84-88. doi: 10.1016/j.foodchem.2008.05.040
- Medina-Godoy, S., Ambriz-Pérez, D. L., Fuentes-Gutiérrez, C. I., Germán-Báez, L. J., Gutiérrez-Dorado, R., Reyes-Moreno, C., & Valdez-Ortiz, A. (2012). Angiotensin-converting enzyme inhibitory and antioxidative activities and functional characterization of protein hydrolysates of hard-to-cook chickpeas. *Journal of the Science of Food and Agriculture*, 92(9), 1974-1981. doi: 10.1002/jsfa.5570
- Montecucco, F., Mach, F., & Pende, A. (2013). Inflammation is a key pathophysiological feature of metabolic syndrome. *Mediators of Inflammation*, 2013. doi: 10.1155/2013/135984
- Montoya, C. A., Lallès, J. P., Beebe, S., & Leterme, P. (2010). Phaseolin diversity as a possible strategy to improve the nutritional value of common beans (*Phaseolus vulgaris*). *Food Research International*, 43(2), 443-449. doi: 10.1016/j.foodres.2009.09.040
- Montoya, C. A., Leterme, P., Victoria, N. F., Toro, O., Souffrant, W. B., Beebe, S., & Lallès, J. P. (2008). Susceptibility of phaseolin to in vitro proteolysis is highly variable across common bean varieties (*Phaseolus vulgaris*). *Journal of Agricultural and Food Chemistry*, 56(6), 2183-2191. doi: 10.1021/jf072576e
- Oseguera-Toledo, M. E., De Mejia, E. G., Dia, V. P., & Amaya-Llano, S. L. (2011). Common bean (*Phaseolus vulgaris* L.) hydrolysates inhibit inflammation in LPS-induced macrophages through suppression of NF-κB pathways. *Food Chemistry*, 127(3), 1175-1185. doi: 10.1016/j.foodchem.2011.01.121

- Przygodzka, M., Zielińska, D., Ciesarová, Z., Kukurová, K., & Zieliński, H. (2014). Comparison of methods for evaluation of the antioxidant capacity and phenolic compounds in common spices. *LWT - Food Science and Technology*, 58(2), 321-326. doi: 10.1016/j.lwt.2013.09.019
- Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, 26(9-10), 1231-1237. doi: 10.1016/S0891-5849(98)00315-3
- Rebello, C. J., Greenway, F. L., & Finley, J. W. (2014). Whole grains and pulses: A comparison of the nutritional and health benefits. *Journal of Agricultural and Food Chemistry*, 62(29), 7029-7049. doi: 10.1021/jf500932z
- Rui, X., Boye, J. I., Simpson, B. K., & Prasher, S. O. (2012). Angiotensin I-converting enzyme inhibitory properties of Phaseolus vulgaris bean hydrolysates: Effects of different thermal and enzymatic digestion treatments. *Food Research International*, 49(2), 739-746. doi: 10.1016/j.foodres.2012.09.025
- Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *Journal of Functional Foods*, 3(4), 229-254. doi: 10.1016/j.jff.2011.05.006
- Torruco-Uco, J., Chel-Guerrero, L., Martínez-Ayala, A., Dávila-Ortíz, G., & Betancur-Ancona, D. (2009). Angiotensin-I converting enzyme inhibitory and antioxidant activities of protein hydrolysates from Phaseolus lunatus and Phaseolus vulgaris seeds. *LWT - Food Science and Technology*, 42(10), 1597-1604. doi: 10.1016/j.lwt.2009.06.006
- Udenigwe, C. C., & Aluko, R. E. (2012). Food protein-derived bioactive peptides: Production, processing, and potential health benefits. *Journal of Food Science*, 77(1), R11-R24. doi: 10.1111/j.1750-3841.2011.02455.x
- Valdez-Ortiz, A., Fuentes-Gutiérrez, C. I., Germán-Báez, L. J., Gutiérrez-Dorado, R., & Medina-Godoy, S. (2012). Protein hydrolysates obtained from Azufrado (sulphur yellow) beans (Phaseolus vulgaris): Nutritional, ACE-inhibitory and antioxidative characterization. *LWT - Food Science and Technology*, 46(1), 91-96. doi: 10.1016/j.lwt.2011.10.021

- Vaz Patto, M. C., Amarowicz, R., Aryee, A. N. A., Boye, J. I., Chung, H. J., Martín-Cabrejas, M. A., & Domoney, C. (2015). Achievements and Challenges in Improving the Nutritional Quality of Food Legumes. *Critical Reviews in Plant Sciences*, 34, 105-143. doi: 10.1080/07352689.2014.897907
- Wan Mohtar, W. A. A. Q. I., Hamid, A. A., Abd-Aziz, S., Muhamad, S. K. S., & Saari, N. (2013). Preparation of bioactive peptides with high angiotensin converting enzyme inhibitory activity from winged bean [*Psophocarpus tetragonolobus* (L.) DC.] seed. *Journal of Food Science and Technology*, 51(12), 3658-3668. doi: 10.1007/s13197-012-0919-1
- Wang, T., He, F., & Chen, G. (2014). Improving bioaccessibility and bioavailability of phenolic compounds in cereal grains through processing technologies: A concise review. *Journal of Functional Foods*, 7(1), 101-111. doi: 10.1016/j.jff.2014.01.033
- World Health Organization (2014). Global status report on noncommunicable diseases 2014. Geneva, Switzerland.
- World Health Organization (2015). Healthy diet. Fact sheet n° 394. (<http://www.who.int/mediacentre/factsheets/fs394/en/>)
- Yeo, J., & Shahidi, F. (2015). Critical evaluation of changes in the ratio of insoluble bound to soluble phenolics on antioxidant activity of lentils during germination. *Journal of Agricultural and Food Chemistry*, 63(2), 379-381. doi: 10.1021/jf505632p
- Zhishen, J., Mengcheng, T., & Jianming, W. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chemistry*, 64(4), 555-559. doi: [http://dx.doi.org/10.1016/S0308-8146\(98\)00102-2](http://dx.doi.org/10.1016/S0308-8146(98)00102-2)
- Zielińska, D., Nagels, L., & Piskula, M. K. (2008). Determination of quercetin and its glucosides in onion by electrochemical methods. *Analytica Chimica Acta*, 617(1-2), 22-31. doi: 10.1016/j.aca.2008.01.037

FIGURE CAPTIONS

Figure 1. Electrophoretic profile of pinto bean proteins hydrolysed by subtilisins at different hydrolysis times. Std, indicate the molecular weights of proteins in the standard Mark12TM (Life Technologies, CA, USA); 0, 30, 60, 90, 120 min are different hydrolysis times. C (-) corresponds to pinto bean protein concentrate.

Figure 2. A) ACE inhibitory activity of 3 kDa permeates from pinto bean hydrolysates (1.5 mg/mL) obtained by subtilisins (Alcalase and Savinase) at different hydrolysis times (0-120 min). C (+) indicate ACE inhibition displayed by 0.1 μ M captopril. B) IL-6 secretion of IL-1 β -stimulated CCD-18Co cells treated by pinto bean hydrolysates for 24 h at 37°C. C(-) refers to cells non-stimulated by IL-1 β . C(+) refers to IL-1 β -stimulated cells non-treated by hydrolysates. Data are expressed as the mean $\pm$ standard deviation of three independent experiments. Different letters indicate significant differences among treatments ($P \leq 0.05$, Duncan test).

Table 1. Peptides (< 3 kDa), total phenolics (TP) and total flavonoids (TF) contents in pinto bean hydrolysates obtained by different subtilisins at different hydrolysis times

Time (min)	<i>ALCALASE</i>			<i>SAVINASE</i>		
	Peptides <3 kDa	TP	TF	Peptides <3 kDa	TP	TF
	(mg/g)	(mg FAE/g)	(mg CAE/g)	(mg/g)	(mg FAE/g)	(mg CAE/g)
0	92.67±4.98 ^a _A	6.14±0.52 ^a _A	3.47±0.30 ^a _A	99.15±4.97 ^a _A	5.22±0.05 ^a _A	3.43±0.24 ^a _A
30	225.30±5.65 ^b _A	11.72±0.23 ^c _B	3.21±0.17 ^a _A	303.52±21.75 ^b _A	10.79±0.26 ^b _A	4.06±0.14 ^{bc} _B
60	344.85±16.77 ^c _A	10.27±0.82 ^b _A	3.71±0.37 ^a _A	352.52±5.65 ^c _A	11.34±0.25 ^b _A	4.42±0.39 ^c _A
90	457.93±23.57 ^d _B	11.59±0.87 ^{bc} _A	3.59±0.07 ^a _A	362.26±5.65 ^c _A	12.93±0.44 ^d _A	4.02±0.20 ^{bc} _A
120	543.78±7.03 ^e _B	12.74±0.73 ^c _A	3.57±0.17 ^a _A	507.96±6.77 ^d _A	12.25±0.28 ^c _A	3.65±0.19 ^{ab} _A

Data indicate mean value ± standard deviation. Different lowercase letters indicate significant differences among hydrolysis times for each enzyme treatment (P<0.05, Duncan test). Different uppercase letters indicate significant differences between enzymatic treatments at the same hydrolysis time (P<0.05, Duncan test).

Table 2. Antioxidant activity of pinto bean hydrolysates obtained by different subtilisins at different hydrolysis times

Hydrolysis time (min)	ORAC (mmol TE/g)	FRAP (μ mol AAE/g)	ABTS (μ mol TE/g)	CV (μ mol TE /g)
<i>ALCALASE</i>				
0	263.04 $\pm$ 18.29 ^a _A	68.90 $\pm$ 0.45 ^b _A	0.36 $\pm$ 0.02 ^a _A	20.66 $\pm$ 0.11 ^a _A
30	308.25 $\pm$ 25.53 ^{bc} _B	66.42 $\pm$ 0.34 ^a _A	0.43 $\pm$ 0.01 ^c _A	25.97 $\pm$ 1.58 ^c _B
60	324.88 $\pm$ 29.16 ^{bc} _A	72.90 $\pm$ 1.13 ^c _A	0.40 $\pm$ 0.02 ^b _A	20.49 $\pm$ 0.69 ^a _A
90	291.63 $\pm$ 21.22 ^b _A	69.54 $\pm$ 1.13 ^b _A	0.43 $\pm$ 0.02 ^c _A	23.11 $\pm$ 0.16 ^b _A
120	348.99 $\pm$ 9.12 ^c _A	64.18 $\pm$ 0.23 ^a _A	0.48 $\pm$ 0.01 ^d _A	22.52 $\pm$ 2.15 ^b _A
<i>SAVINASE</i>				
0	233.47 $\pm$ 10.96 ^a _A	75.46 $\pm$ 0.68 ^b _A	0.40 $\pm$ 0.01 ^a _A	18.15 $\pm$ 0.88 ^a _A
30	262.67 $\pm$ 26.34 ^b _A	71.06 $\pm$ 0.34 ^{ab} _B	0.50 $\pm$ 0.02 ^b _B	18.14 $\pm$ 1.55 ^a _A
60	299.38 $\pm$ 21.57 ^c _A	70.82 $\pm$ 1.36 ^{ab} _A	0.50 $\pm$ 0.01 ^b _B	20.56 $\pm$ 0.84 ^a _A
90	318.25 $\pm$ 19.46 ^c _B	69.62 $\pm$ 0.34 ^{ab} _A	0.49 $\pm$ 0.02 ^b _B	24.08 $\pm$ 0.40 ^b _A
120	326.11 $\pm$ 30.18 ^c _A	67.46 $\pm$ 3.17 ^a _A	0.49 $\pm$ 0.01 ^b _A	25.94 $\pm$ 0.16 ^b _A

Data indicate mean value $\pm$ standard deviation. Different lowercase letters indicate significant differences among hydrolysis times for each enzyme treatment ($P < 0.05$, Duncan test). Different uppercase letters indicate significant differences between enzymatic treatments at the same hydrolysis time ($P < 0.05$, Duncan test).

Table 3. Most abundant peptides found in the most active pinto bean hydrolysates.

ALCALASE, 120 min			SAVINASE, 90 min		
Protein	Peptide sequence ¹	MH+ [Da]	Protein	Peptide sequence	MH+ [Da]
α -type phaseolin precursor, Phaseolin	SIEMKEGAL FVPH	1457.75	α -type phaseolin precursor, Phaseolin	SIEMKEGAL FVPH	1457.75
α -type phaseolin precursor, Phaseolin	VNPDPKEDLRRIQL	1649.92	α -type phaseolin precursor	VV NEGEAH VELVGPKGNKETLE FESY	2874.42
α -type phaseolin precursor, Phaseolin	DNVISSIGRAL	1144.63	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	YLVNPDPKEDLRRIQL	1926.07
α -type phaseolin precursor	VV NEGEAH VELVGPKGNKETLE FESY	2874.41	β -type phaseolin storage protein	STEAQQSYLQEFESKHILEAS	2296.11
α -type phaseolin precursor, Phaseolin	STEAQQSYLQEFESKHIL	2009.00	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	SIEMEEGAL FVPH	1458.69
α -type phaseolin precursor	GDNPIFSDNQKIPAGTIF	1933.97	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	VNPDPKEDLRRIQL	1649.92
α -type phaseolin precursor, Phaseolin	SQDNPFYFNSDNSWNTL	2048.86	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	FNSKFEEINRVL	1495.79
α -type phaseolin precursor, Phaseolin	DNVISSIGRALDGKDVGL	1942.06	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	TSLREEEESQDNPFYFN	2104.91
α -type phaseolin precursor, Phaseolin	ISSIEMKEGAL FVPH	1657.86	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	VV NEGEAH VELVGPKGNKETLE YESY	2890.41
α -type phaseolin precursor, Phaseolin	YLVNPDPKEDLRRIQL	1926.07	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	QADAELLVRS	1313.74
Phaseolin	SDNPIFSDH Q KIPAGTIF	1986.99	Phaseolin	AMPVNNP Q IHDFFLS	1729.84
Phaseolin	VV NEGEAH VELVGPKGNKETLE YESY	2890.41	α -type phaseolin precursor, β -type phaseolin storage protein	AMPVNNP Q IHEFFLS	1743.85
α -type phaseolin precursor	AMPVNNP Q IHEFFLS	1743.85	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	SIGRALDGKDVGL	1413.81
α -type phaseolin precursor, Phaseolin	RAELSKDDVFV IP AAYPAIK	2302.28	α -type phaseolin precursor	GDNPIFSDNQKIPAGTIF	1933.97
α -type phaseolin precursor, Phaseolin	SIEMKEGAL FVPHYYSK	1999.00	Phaseolin, β -type phaseolin storage protein	SDNPIFSDH Q KIPAGTIFYL	2263.15
Phaseolin	AMPVNNP Q IHDFFLS	1729.84	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	SLREEEESQDNPFYFN	2003.86
α -type phaseolin precursor, Phaseolin	SQDNPFYFNSDNSWNTL FKN	2438.07	Phaseolin, β -type phaseolin storage protein	TSDNPIFSDH Q KIPAGTIF	2088.04
α -type phaseolin precursor, Phaseolin	SDNSWNTL FKN	1325.61	α -type phaseolin precursor, β -type phaseolin storage protein	FE EEGQQEEGQQEGVIV	1934.87
Phaseolin	TSDNPIFSDH Q KIPAGTIFYL	2364.19	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	SDNSWNTL FKN QYGH IR VL	2292.15
α -type phaseolin precursor, Phaseolin	SDNSWNTL FKN QYGH IR	2080.00	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	DNVISSIGRALDGKDVGL	1942.06
α -type phaseolin precursor	GDNPIFSDNQKIPAGTIFYL	2210.12	Phaseolin, β -type phaseolin storage protein	SDNPIFSDH Q KIPAGTIF	1986.99
Phaseolin	SDNPIFSDH Q KIPAGTIFYL	2263.14	β -type phaseolin storage protein	ISSIEMEEGAL FVPH	1658.81
α -type phaseolin precursor, Phaseolin	VV NEGEAH VE	1082.51	α -type phaseolin precursor	GDNPIFSDNQKIPAGTIFYL	2210.12

α -type phaseolin precursor, Phaseolin	SDNSWNTLFKNQYGHIRVL	2292.15	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	SKRLQNLEDYRLVEF	1910.01
α -type phaseolin precursor, Phaseolin	ISSIEMKEGALFVPHYYSK	2199.11	Phaseolin, β -type phaseolin storage protein	TSDNPIFSDHQKIPAGTIFYL	2364.19
α -type phaseolin precursor, Phaseolin	LSKDDVVFVIPAAYPVAIK	1946.10	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	RAELSKDDVVFVIPAAYPVAIK	2302.28
α -type phaseolin precursor, Phaseolin	VVNEGEAHVELVGPK	1576.83	α -type phaseolin precursor	LVGPKGNKETLEFESY	1810.92
α -type phaseolin precursor	SGSYFVDGHHHQEQQKGS HQE	2678.17	α -type phaseolin precursor, Phaseolin	SIEMKEGALFVPHYYSK	1999.00
α -type phaseolin precursor	SGSYFVDGHHHQEQE	1627.69	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	ILEASFNSKFEEINRVL	2009.07
α -type phaseolin precursor	KQSGSYFVDGHHHQEQQKGS HQE	2934.32	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	LLPQQADAELLVVR	1677.99
α -type phaseolin precursor	INKQSGSYFVDGHHHQEQQKGS H	2904.35	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	YLVNPDPKEDLR II	1684.93
Phaseolin	GQQEGVIVNIDSEIQE	1885.95	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	LLPQQADAELLVVR S	1765.02
α -type phaseolin precursor, Phaseolin	LSKDDVVFVIPAAYPVAIKAT	2118.19	α -type phaseolin precursor, Phaseolin, β -type phaseolin storage protein	VVNEGEAHVELVGPK	1576.83
α -type phaseolin precursor, Phaseolin	DNSWNTLFKNQYGHIRVL	2205.12	β -type phaseolin storage protein	SIEMEEGALFVPHYYSKAI VIL	2509.31
α -type phaseolin precursor, Phaseolin	VNPDPKEDLR II	1408.78			
α -type phaseolin precursor, Phaseolin	YLVNPDPKEDLR II	1684.93			
α -type phaseolin precursor	TQGDNPIFSDNQKIPAGTIF	2163.07			

[†] Underlined, Bolded, and highlighted portion of the sequence found to be part of the peptide with reported antioxidant, ACE inhibitory and anti-inflammatory activity, respectively (BIOPEP database; Oseguera-Toledo et al., 2011). PSMs

Table 4. Phenolic profile (µg/g DW) of the most active pinto bean hydrolysates

	CONTROL	ALCALASE, 120 min	SAVINASE, 90 min
<i>p</i> -Coumaric acid	1.00±0.01 ^a	1.01±0.03 ^a	1.13±0.11 ^a
Sinapic acid	0.21±0.01 ^a	0.19±0.01 ^a	0.24±0.04 ^a
Ferulic acid	28.24±0.08 ^a	26.41±0.23 ^a	35.23±5.75 ^b
<i>Total hydroxycinnamic acids</i>	26.06±0.41 ^a	27.62±0.19 ^a	59.88±5.07 ^b
Catechin	41.24±1.48 ^a	43.61±2.69 ^a	72.50±1.42 ^b
Epicatechin	15.23±0.11 ^c	11.86±0.21 ^a	13.49±0.19 ^b
<i>Total flavanols</i>	56.47±1.59 ^a	55.46±2.91 ^a	85.99±1.61 ^b
Quercetin 3-glucoside	8.28±0.42 ^a	8.08±0.28 ^a	9.98±1.16 ^a
Quercetin	1.60±0.05 ^a	1.70±0.11 ^a	1.78±0.27 ^a
Isorhamnetine 3-rutinoside	0.81±0.03 ^a	0.80±0.01 ^a	0.92±0.11 ^a
Myricetin 3-glucoside	0.75±0.05 ^a	0.70±0.01 ^a	0.76±0.01 ^a
Quercetin 3-xylosylglucoside	0.79±0.00 ^a	0.77±0.02 ^a	0.96±0.07 ^b
Quercetin 3-malonylglucoside	1.87±0.06 ^c	1.37±0.00 ^a	1.66±0.01 ^b
Kaempferol 3-malonylglucoside	1.10±0.03 ^a	0.96±0.02 ^a	1.07±0.09 ^a
<i>Total flavonols</i>	15.20±0.48 ^a	14.37±0.41 ^a	17.13±1.72 ^a
Cyanidin 3-glucoside	1.92±0.00 ^a	1.98±0.06 ^a	1.93±0.07 ^a
Cyanidin	0.87±0.01 ^a	0.96±0.01 ^b	0.94±0.04 ^{ab}
Cyanidin 3-rutinoside	0.75±0.00 ^{ab}	0.73±0.01 ^a	0.85±0.06 ^b
Delphinidin 3-glucoside	0.91±0.00 ^a	0.91±0.04 ^a	0.98±0.11 ^a
Pelargonidin diglucoside	1.08±0.00 ^a	1.05±0.02 ^a	1.34±0.15 ^a
Malvidin 3-glucoside	0.69±0.01 ^a	1.07±0.04 ^b	1.07±0.07 ^b
Pelargonidin 3-glucoside	16.11±0.44 ^a	14.40±0.06 ^a	49.33±4.59 ^b
Cyanidin 3-malonylglucoside	1.10±0.01 ^a	1.04±0.04 ^a	1.13±0.07 ^a
Pelargonidin 3-malonylglucoside	2.63±0.01 ^c	1.64±0.05 ^a	2.31±0.09 ^b
<i>Total anthocyanins</i>	26.06±0.41 ^a	23.77±0.17 ^a	59.88±5.07 ^b
Total phenolics	127.18±2.58^a	121.22±2.52^a	199.61±14.22^b

Data indicate mean value ± standard deviation. Different letters within a row are significantly different

(P<0.05, Duncan test).

Figure 1

Figure 2

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Supplementary Table 1.

Compound	Q1 (Da)	Q3 (Da)	declustering potential (V)	collision energy (V)	collision cell exit potential (V)
<u>positive ionization</u>					
cyanidin	287.0	137.0	180	46	12
cyanidin 3-glucoside	449.0	287.0	100	25	20
cyanidin 3-rutinoside	595.0	287.0	100	35	23
cyanidin 3-malonylglucoside	535.0	287.0	100	30	23
delphinidin 3-glucoside	465.0	303.0	100	30	23
pelargonidin diglucoside	595.0	271.0	100	30	23
pelargonidin 3-glucoside	433.0	271.0	100	30	23
pelargonidin 3-malonylglucoside	519.0	271.0	100	30	23
malvidin 3-glucoside	493.0	331.0	100	30	23
<u>negative ionization</u>					
ferulic acid	193.0	134.1	-180	-24	-12
<i>p</i> -coumaric acid	162.9	119.0	-90	-20	-12
sinapic acid	223.0	200.1	-100	-21	-20
catechin	289.2	245.3	-100	-22	-16
epicatechin	289.2	245.3	-100	-22	-16
quercetin	301.1	151.1	-115	-35	-12
quercetin 3-glucoside	463.0	301.0	-100	-40	-15
quercetin 3-xylosylglucoside	595.0	301.0	-100	-40	-15
quercetin 3-malonylglucoside	549.0	301.0	-100	-40	-15
isorhamnetin 3-rutinoside	623.0	315.0	-100	-40	-15
kaempferol 3-malonylglucoside	533.0	285.0	-100	-40	-15
myricetin-3-glucoside	479.0	317.0	-100	-40	-15

Supplementary Table 2.

ion source conditions	ionization mode	
	positive	negative
nitrogen curtain gas	25 L/min	25 L/min
collision gas	9 L/min	9 L/min
ion spray source voltage	5400 V	-4500 V
temperature	350 °C	350 °C
nebulizer gas	35 L/min	35 L/min
turbo gas	30 L/min	30 L/min