Germination of *Phaseolus vulgaris* and alcalase hydrolysis of its proteins produced bioactive peptides capable of improving markers related to type-2 diabetes *in vitro*

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17 **Abstract**

18 The aim of this study was to evaluate the effect of germination and alcalase hydrolysis of common
19 bean proteins on the generation of bioactive peptides with potential to reduce parameters related to
20 the risk of developing type-2 diabetes (T2D) *in vitro*. Germination (25°C up to 72 h) and alcalase
21 hydrolysis (up to 4 h) produced peptides with high antioxidant capacity (1085 µmol TE/g soluble
22 protein, SP). After 24 h of germination, there was an increase of 44% in α -amylase inhibitory
23 capacity of the peptides relative to acarbose (1 mM). Simulated gastrointestinal digestion of the non-
24 germinated and non-hydrolyzed sample (G0-0h) produced bioactive peptides that inhibited
25 dipeptidyl peptidase-IV (DPP-IV) (IC_{50} =1.2 mg SP/mL); however, the IC_{50} was not improved by
26 either germination or alcalase hydrolysis. Insulin secretion by glucose stimulated (20 mM) INS-1E
27 pancreatic β -cells, increased 45% from the basal state with 2 mg SP/mL of G0-0h. Germination did
28 not improve the stimulation of insulin. Computational modeling showed that phaseolin fraction
29 RGPLVNPDPKPFL obtained after 48 h germination and 1 h alcalase-hydrolysis was able to inhibit
30 DPP-IV by interacting with its S1, S2, and S3 pockets of the active site. Germination and alcalase
31 hydrolysis can be applied to improve some markers related to the management of T2D. Furthermore,
32 simulated gastrointestinal digestion of common bean proteins without any treatment produced
33 bioactive peptides with the ability to inhibit DPP-IV, resulting in increased insulin released from
34 pancreatic cells *in vitro*.

35

36 **Keywords:** Alcalase, Common bean, Germination, Pancreatin, Pepsin, type-2 Diabetes

37 1. Introduction

38 Common bean (*Phaseolus vulgaris* L.) is a legume widely consumed around the world. It is
39 one of the most important staples in the Brazilian diet. Brazil is the second highest producer of
40 common beans in the world and according to Brazilian Ministry of Agriculture, it is predicted a
41 consumption of 16 kg per capita of common beans in 2014, with a production of 3.3 millions of tons
42 (CONAB, 2013). Common bean is rich in proteins (15 to 35%), which are mainly globulins (45 –
43 70%) and albumins (10 – 30%) (Sathe, 2002; Boye, Zare, & Pletch, 2010). The most abundant
44 protein in common beans is phaseolin (Montoya, Lallès, Beebe, & Leterme, 2010). Several anti-
45 nutritional compounds are also present in common beans, such as protease inhibitors Kunitz and
46 Bowman Birk, and α -amylase inhibitor (Sathe, 2002).

47 Some studies have associated the consumption of common beans to the decreased risk of
48 cardiovascular disease, diabetes, obesity, and cancer (Bazzano et al., 2001; Aparicio-Fernandez et
49 al., 2006; Díaz-Batalla, Widholm, Fahey, Castaño-Tostado, & Paredes-López, 2006; Vital, de Mejia,
50 Dia, & Loarca-Piña, 2014; Mojica, & de Mejia E.G. (2015)). In the past fifteen years, there has been
51 a 374% increase in the number of people with type-2 diabetes (T2D) in Brazil, and currently there
52 are around 12 million people in the country living with T2D (DATASUS, 2014).

53 Health effects of common beans are often related to the content of dietary fiber and
54 phytochemicals, including polyphenolic compounds. However, it has also been shown that proteins
55 are able to act as antioxidants, to prevent cellular damage caused by reactive free radical that may
56 initiate diseases such as atherosclerosis, arthritis, diabetes and cancer (Sarmadi & Ismail, 2010).
57 Other interesting health-related properties of common bean can be obtained from the proteins after
58 hydrolysis, mainly by the action of enzymes (Sarmadi & Ismail, 2010, Kong, Guo, Hua, Cao, &
59 Zhang, 2008). Hydrolysis generates small peptides and free amino acids that are absorbed in the
60 gastrointestinal tract. Based on their structural properties, their amino acid composition and
61 sequence, these peptides may play various biological roles, such as antihypertensive,

immunomodulators, antimicrobials, antithrombotic, and antidiabetic (Sarmadi & Ismail, 2010; Kannan, Hettiarachchy, & Marshall, 2012; Malaguti et al., 2014), and for that, they are called bioactive peptides.

Bioactive peptides can also be produced during seed germination. This is an inexpensive and effective process that can be used to increase the nutritional value of common beans by improving the protein digestibility and decreasing anti-nutritional compounds (Barcelos, Vilas Boas, & Lima, 2002; Vernaza, Dia, de Mejia, & Chang, 2012; Aguilera et al., 2013). During germination, several biochemical mechanisms are activated, such as hydrolysis of starch and proteins, in order to meet the needs of the new plant (Klose & Arendt, 2012). Endopeptidases degrade storage proteins producing oligopeptides, which are further degraded by exopeptidases forming free amino acids (Zakharov et al., 2004; Sangronis, Rodríguez, Cava, & Torres, 2006).

Several chronic non-transmissible diseases may potentially be prevented by using bioactive peptides as a functional food, dietary supplement or nutraceutical (Sarmadi & Ismail, 2010; Boschini, Scigliuolo, Resta, & Arnoldi, 2014). The consumption of bioactive peptides obtained from common beans may prevent the risk of T2D and could be used as an alternative to help combat this disease. The objective of this study was to evaluate the effect of germination of common bean (*Phaseolus vulgaris*) and alcalase hydrolysis of its proteins to produce and identify bioactive peptides with the potential to reduce markers related to T2D, after *in vitro* simulated gastrointestinal digestion.

2. Materials and methods

2.1 Materials

Common bean (*Phaseolus vulgaris*), from the black seed coat IAC-UNA cultivar, were provided by Agronomical Institute of Campinas (IAC) (Campinas, BR). The enzymes used were: alcalase from *Bacillus licheniformis* (EC 3.4.21.62; 2.4 U/g, U=amount of enzyme that catalyzes the conversion of 1 μ mol of substrate per min, under specified conditions); porcine pepsin (EC

3.4.23.1); pancreatin (EC 232-468-9, a mixture of several digestive enzymes produced by exocrine cells of porcine pancreas); recombinant human dipeptidyl peptidase IV (DPPIV, EC 3.4.14.5, ≥ 1.0 U/vial) ; and α -amylase from porcine pancreas (EC 3.2.1.1, ≥ 10 U/mg solid, type VI-B). Bovine serum albumin (BSA, >98% pure). The antibodies used were: Bowman Birk Inhibitor (BBI) primary antibody, epitope: 238 I mouse monoclonal antibody (Agdia, Inc., Elkhart, IN); and for Kunitz Trypsin Inhibitor (KTI) was trypsin inhibitor rabbit polyclonal antibody, epitope: full length protein (Abcam, Cambridge, MA). Antimouse IgG alkaline phosphatase conjugate (Sigma- Aldrich, St. Louis, MO) was used as the secondary antibody for BBI, and for KTI was antirabbit IgG alkaline phosphatase conjugate. DPPIV-GLO protease assay kit, a homogeneous, luminescent assay that measures dipeptidyl peptidase IV (DPPIV) activity, was purchased from Promega (Madison, WI). INS-1E cells were kindly provided by Dr. Maechler from the Division of Clinical Biochemistry, Department of Internal Medicine, University Medical Center, Switzerland. The Insulin Human ELISA kit was acquired from Abcam (Cambridge, MA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.2 Preparation of common bean germinated flour and concentration of the proteins

Common beans (500 g) were sanitized for 10 min with sodium hypochlorite (100 mg/kg) and then were rinsed 3 times with distilled water. They were placed into a germination chamber using a tray covered with germination paper. The germination was carried out for 0, 24, 48, or 72 h at 25°C in a light-darkness cycle (12/12 h) and at a 100% relative humidity inside the chamber; the germination of common bean was performed in triplicate **Figure 1A** shows the resulting germinated common beans. Common beans were manually peeled, frozen at -20°C, freeze-dried (L108, Liobras), and grounded to produce flour with particle size equivalent to 20 mesh. To produce the common bean protein concentrate (Oseguera-Toledo, de Mejia, Dia, & Amaya-Llano, 2011), 300 g of the flour were suspended in water and the pH was adjusted to 8.0 by adding 0.2 M NaOH. The extraction was carried out at 35°C with agitation for 1 h. The suspension was centrifuged at 5000 g

112 for 15 min at 25°C. The supernatant had its pH adjusted to 4.3 with 1.0 M HCl. Then, the suspension
113 was centrifuged at 10,000 g for 20 min. The pellet was frozen at -20°C, freeze-dried (L108, Liobras),
114 and kept at -20°C until utilization.

115 *2.3 Preparation of alcalase hydrolysates from protein concentrates*

116 Common bean protein concentrates were hydrolyzed using alcalase. Based on preliminary
117 experiments (data not shown) the following parameters were selected. A suspension of 8 g of protein
118 concentrate in 100 mL of water was prepared and the pH was adjusted to 8.0 using 0.5 M NaOH.
119 The suspension was incubated at 50°C in a water bath for 10 min and then 2.5 mL (0.75 U/g of
120 protein) of alcalase was added. The samples were hydrolyzed for 0, 1, 2, 3 or 4 h and the pH was
121 maintained at 8.0 by adding 0.5 M NaOH. The hydrolysis was stopped by adding 1.2 mL of 0.1 M
122 HCl. The hydrolysates were centrifuged at 14,000 g for 30 min at 10°C. The supernatants were
123 ultrafiltered through a 0.8 kDa membrane in order to remove salts. The protein hydrolysates were
124 frozen at -20°C, freeze-dried (L108, Liobras) and maintained at -20°C.

125 *2.4 Soluble protein*

126 Soluble protein (SP) concentration of the protein hydrolysates was determined using Protein
127 DC Microplate Assay from Bio-Rad (Hercules, USA). The extraction and purification of the proteins
128 from non-germinated and germinated common beans were described in **section 2.2**. Briefly, 5 µL of
129 common bean samples (2.0 mg/mL) diluted in water (pH=7.0) were placed in 96 well plate, mixed
130 with 25 µL of reagent A (alkaline copper tartrate solution) and 200 µL of reagent B (diluted Folin
131 reagent). The plate was allowed to stand for 15 min at room temperature and the absorbance was
132 read at 630 nm. A standard curve of pure bovine serum albumin (BSA) was prepared using
133 concentrations of 0.010-1.5 mg/mL in buffer solution and measured along with samples for each run.
134 The protein concentration was calculated using BSA standard curve ($y = 0.0001x - 0.0017$,
135 $R^2=0.99$).

136 2.5 Antioxidant capacity

137 Common bean samples were extracted and prepared as mentioned in **sections 2.2 and 2.3**. The
138 freeze-dried bean samples were diluted in 75 mM phosphate buffer, pH 7.4, to the final concentration
139 of 2.0 mg/mL. Antioxidant capacity was measured by the oxygen radical absorbance capacity
140 (ORAC) assay (Prior & Cao, 1999). A comparative evaluation was performed using 20 μ L of
141 diluted samples, trolox standard (6-hydroxy-2,5,7,8-etramethylchroman 2-carboxylic acid, 1-8 μ M
142 final concentration), or blank (75 mM phosphate buffer, pH 7,4); 120 μ L of 116,9 nM fluorescein
143 (final concentration of 70 nM/well), and 60 μ L of 40 mM AAPH [Azobis (2-aminodinopropane)
144 dihydrochloride] per well. A black walled 96-well plate was read at excitation wavelength of 485 nm
145 and an emission wavelength of 528 nm every 2 min and sensitivity 60 at 37°C using a Fluostar
146 Omega plate reader (BMG Labtech, Ortenberg, Germany). Results were expressed as μ mol Trolox
147 equivalents (TE)/g hydrolyzed sample and also as μ mol TE/g SP, using a standard curve ($y =$
148 $0.0421x - 0.1586$, $R^2 = 0.99$).2.6 *Quantification of Bowman-Birk inhibitor, Kunitz trypsin inhibitor,*
149 *and α -amylase inhibitor*

150 Bean samples were suspended in water (1 mg/mL) and diluted 1:1 in TBS (Tris-buffered
151 saline). One hundred microliters of samples suspensions were plated in a 96-well plate and store
152 overnight at 4°C. After blocking with 300 μ L 5% BSA in TBS with 1% Tween 20 for 1 h, 50 μ L of
153 the primary and secondary antibodies were added and incubated during 1 h each. After each step, 5-
154 wash cycles with PBS buffer were performed. A color reaction occurred after adding 100 μ L of 4-
155 nitrophenyl phosphate disodium salt hexahydrate (pNPP) and incubating overnight at 4°C. The
156 reaction was stopped by adding 100 μ L of 3 N NaOH. After 25 min, the absorbance was read at 405
157 nm. The primary antibody dilution for BBI was 1:200 using BBI mouse monoclonal antibody (Agdia
158 Inc., Elkhart, IN) and for KTI was 1: 3000 using trypsin inhibitor rabbit polyclonal (Abcam,
159 Cambridge, MA). As a secondary antibody, antimouse IgG alkaline phosphatase conjugate (1:2000)
160 was used for BBI, and antirabbit IgG alkaline phosphatase conjugate (1:1000) was used for KTI.

161 Standard curves were determined using purified BBI (70–90% purity) and purified KTI (>98%
162 purity). Each sample was analyzed in triplicate.

163 Alpha-amylase inhibition was performed by adding 500 μ L of bean sample suspensions (1
164 mg/mL) or positive control (1 mM acarbose) to 500 μ L of 13 U/mL α -amylase solution (type VI-B
165 from porcine pancreas in 0.02 M sodium phosphate buffer pH 6.9) and incubating in test tubes at
166 25°C for 10 min. Then, 500 μ L of 1% soluble starch solution (dissolved in sodium phosphate buffer
167 and boiled for 15 min) was added to each tube and incubated for another 10 min. Finally, 1 mL of
168 dinitrosalicylic acid color reagent was added and the tubes were placed in 100°C water bath for 5
169 min. The mixture was diluted with 10 mL of distilled water and absorbance was read at 520 nm.
170 Results were presented as percent inhibition relative to the positive control acarbose (1 mM), which
171 had 100% inhibition.

172 2.7 Simulated gastrointestinal digestion

173 In order to simulate *in vitro* digestion of the bean protein hydrolysates (Megías et al., 2009),
174 these were suspended in water (1:20 w/v) and a sequential enzyme digestion was carried out with
175 pepsin (enzyme: substrate 1:20 w/w) at 37°C, pH 2.0 for 2 h, followed by pancreatin (enzyme:
176 substrate 1:20 w/w) at 37°C, pH 7.5 for 2 h. The digestion was stopped by heating the samples to 75
177 °C for 20 min. The samples were centrifuged at 20,000 g for 15 min at 4°C and the supernatant were
178 frozen at -20°C, freeze-dried (L108, Liobras) and maintained at -20°C.

179 2.8 SDS-PAGE electrophoresis of common bean protein hydrolysates

180 All protein alcalase-hydrolysates were diluted (20 mg/mL) in Laemmli buffer containing 5%
181 of β -mercaptoethanol and boiled for 5 min prior loading (15 μ L). The bands of the proteins were
182 analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 4-20%
183 Tris-HCl ready gels (Bio-Rad, Hercules, CA). A prestained Precision Plus Protein Standard (Bio-
184 Rad, Hercules, CA) was used. The gel was run at 200 V, 10 mA, for 35 min. After staining with

185 SimplyBlue Safe Stain (Invitrogen, Carlsbad, USA) overnight, and destaining with water, the gels
186 were visualized using GL 4000 Pro Imaging System (Carestream Health Inc., Rochester, NY). The
187 samples digested by pepsin and pancreatin were diluted (50 mg/mL) in tricine sample buffer (Bio-
188 Rad, Hercules, CA) containing 5% of β -mercaptoethanol and boiled for 5 min prior loading (15 μ L).
189 A polypeptide SDS-PAGE standard diluted 1:1 in tricine sample buffer (Bio-Rad, Hercules, CA)
190 containing 5% of β -mercaptoethanol was used. The bands of the peptides were analyzed by SDS-
191 PAGE using 16.5% Tris-Tricine polypeptide ready gels (Bio-Rad, Hercules, CA). The Polypeptide
192 SDS-PAGE Standard (Bio-Rad, Hercules, CA) was used. The gels were run for 240 min using Tris-
193 Tricine-SDS buffer with the conditions set at 65 mA constant (60 -125V). The gels were stained with
194 SimplyBlue Safe Stain (Invitrogen, Carlsbad, CA) overnight, fixed for 30 min in methanol/acetic
195 acid/ water (10:40:50%) and destaining with acetic acid/water (40%) for 1 h, and water for 8 h,
196 changing the water every hour. The gels were visualized using GL 4000 Pro Imaging System
197 (Carestream Health Inc., Rochester, NY).

198 *2.9 Characterization of the peptides and sequence identification*

199 The peptides obtained after simulated gastrointestinal digestions were diluted in deionized
200 water (100 μ g/mL) and they were analyzed by high-performance liquid chromatography/electrospray
201 ionization tandem mass spectrometry (HPLC-ESI-MS) using a Q-tof Ultima mass spectrometer
202 (Waters, Milford, USA), equipped with an Alliance 2795 HPLC system. The separation of the
203 components was performed by using a mobile phase of solvent A (95% H₂O, 5% ACN and 0.1%
204 Formic Acid) and solvent B (95% ACN, 5% H₂O and 0.1% formic acid) in a flow rate of 200
205 μ L/min. The elution was in a linear gradient (0 min, 90% A; 2 min, 90% A; 40 min, 65% A; 60 min,
206 10% A; 65 min, 10% A; 66 min, 90% A; 80 min, 90% A). The temperature was kept at 20°C during
207 the whole procedure. A splitter with a split ratio of 1:10 was used, where one part went to the mass
208 spectrometer and ten parts went to waste. The Q-tof Ultima mass spectrometer was equipped with a
209 Z-spray ion source. Using a positive ion electrospray mode (+ESI), the analysis on the Q-tof was

carried out in V-mode with an instrument resolution between 9,000 and 10,000 based on full width at half maximum, with a flow rate of 20 μ L/min. The source temperature was set at 80°C and desolvation temperatures were set at 250°C, respectively. The Q-tof was operated at capillary voltage of 3.5 kV and a cone voltage of 35V. The final detector was a microchannel plate with high sensitivity. The MassLynx 4.1V software (Waters, Milford, MA) was used to control the instruments and to process the data in order to get the highest probability of the peptides sequences. The presence of the peptides in common bean (*Phaseolus vulgaris*) was confirmed using BLAST[®] tool (<http://www.blast.ncbi.nlm.nih.gov/Blast.cgi>, accessed in 04/26/2014). The potential biological activity of the peptides was predicted by using BIOPEP database (<http://www.uwm.edu.pl/biochemia>, accessed in 04/26/2014).

2.10 DPP-IV inhibition biochemical assay

DPP-IV inhibition was measured using the DPP-IV Glo Protease Assay (G8351, Promega, Madison, WI) following the manufacture's protocol. Briefly, 50 μ L of DPP-IV Glo reagent was added to a white-walled 96-well plate containing either 50 μ L of blank, 40 μ L enzyme control or 40 μ L sample. Bean samples were prepared in an assay buffer (100 mM Tris, pH 8.0, 200 mM NaCl, 1 mM EDTA) at concentrations of 0.1, 0.5, 1.0, 2.0, or 4.0 mg/mL. The blank contained only assay buffer, while the enzyme control and the samples contained assay buffer and 10 μ L purified DPP-IV human enzyme (100 ng/mL). Luminescence was then measured after gentle mixing and incubation at 26°C for 30 min using a Synergy2 multiwell plate reader (Biotek Instruments, Winooski, VT). Percent inhibition was calculated from the blank and enzyme control for each sample. IC₅₀ was calculated from the quadratic formula generated from the log (concentration) and percent inhibition.

2.11 INS-1E cells treatment for glucose-stimulated insulin secretion

In order to understand the role of peptides obtained after simulated gastrointestinal digestion on insulin released from INS-1E cells, glucose-stimulated insulin secretion was measured (Merglen et

234 al. 2004). INS-1E cells (passage 58-59) were cultured in Roswell Park Memorial Institute (RPMI)
235 1640 supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2
236 mM L-glutamine, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, and 4500 mg/L glucose. INS-
237 1E cell were plated (8×10^4 cells/well in 24-well plates) and incubated for 3 days at 37°C, in a
238 humidified 95% air, 5% CO₂ atmosphere. Thereafter, the cells were treated by adding the
239 supplemented RPMI 1640 media containing 2 mg/mL of the samples and incubating for 24 h. Two
240 hours before the glucose stimulation the media was replaced by RPMI 1640 without glucose
241 supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% HEPES, 1% sodium
242 pyruvate, 600 μ L β -mercaptoethanol and incubated again for 2 h as starvation. The cells were
243 washed 3 times with modified Krebs-Ringer-bicarbonate-HEPES buffer (KRBH), containing 135
244 mM NaCl, 3.6 mM KCl, 0.5 mM NaH₂PO₄, 0.5 mM MgCl₂, 10 mM HEPES, 2 mM NaHCO₃, and
245 1.5 mM CaCl₂, and were incubated for 30 min. The KRBH was replaced by KRBH with 0.1% of
246 bovine serum albumin (BSA) and 0 or 20 mM glucose for glucose stimulation during 30 min of
247 incubation. A KRBH with BSA and without glucose was used as a negative control. The supernatant
248 was collected, centrifuged at 13,000 rpm for 1 min and was stored at -20°C. The insulin secretion
249 measurement was performed utilizing an Insulin Human ELISA kit (Abcam, Cambridge, MA).
250 Briefly, 100 μ L of standard (recombinant human insulin, 0-300 μ IU/mL) or sample were placed into
251 the wells of the provided plate and incubated at room temperature for 2.5 h. The wells were washed 4
252 times with the wash solution provided. Then, 100 μ L of biotinylated insulin detection antibody were
253 added to each well and the plate was incubated for 1 h at room temperature and washed again. A 100
254 μ L of horseradish peroxidase (HRP)-streptavidin solution was added to each well and the plate was
255 incubated for 45 min at room temperature. The plate was washed again and incubated for 30 min at
256 room temperature with 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate one-step
257 reagent in each well. A 50 μ L of the stop solution was added to each well and the plate was read

258 immediately at 450 nm on an ELx808™ Microplate Reader (Biotek Instruments, Winooski, VT).
259 Insulin content was calculated using the standard curve $y = 0.0014x - 0.0068$, $R^2 = 0.986$.

260

261 *2.12 Computational modeling*

262 The structure of DPP-IV was obtained from the Research Collaboratory for Structural
263 Bioinformatics (RCSB) Protein Data Bank (PDB) file 1X70, in which DPP-IV exists as dimer. One
264 monomer of DPP-IV was deleted from the file using Accelrys Discovery Studio 4.0 Visualizer (DS
265 4.0, Accelrys Software Inc., Discovery Studio Modeling Environment, Release 4.0, San Diego:
266 Accelrys Software Inc., 2013.) as only one monomeric unit was necessary to characterize
267 interactions with DPP-IV. Water molecules and extra ligands present in PDB file 1X70 were deleted,
268 and the remaining DPP-IV monomer was added hydrogen atoms using DV 4.0. The structure of the
269 peptide sequence RGPLVNPDPKPFL was obtained by requesting the folding of the peptide
270 sequence through Pepstr: Peptide tertiary structure prediction server in a vacuum environment (Kaur,
271 Garg, & Raghava, 2007). The folded peptide was added hydrogen atoms using DV 4.0, and was then
272 docked to the prepared monomeric unit of DPP-IV using ClusPro protein-protein docking server
273 (vers 2.0) (Comeau, Gatchell, Vajda, & Camacho, 2004; Kozakov et al., 2013; Kozakov, Brenke,
274 Comeau, & Vajda, 2006). The best peptide conformation from ClusPro was determined to be that
275 which had the highest cluster size for balanced coefficients. The DPP-IV complex that included this
276 conformation was saved in PDB format. Hydrogen atoms were removed from the peptide
277 conformation in the complex using DV 4.0. The DPP-IV complex obtained from ClusPro was then
278 sent to the Rosetta FlexPepDock web server to have the peptide conformation refined (London,
279 Raveh, Cohen, Fathi, & Schueler-Furman, 2011; Raveh, London, & Schueler-Furman, 2010). The
280 default of 100 high and low resolution structures to be made per run was kept. Rosetta FlexPepDock
281 web server has problems analyzing hydrogen atoms in proline amino acids in peptides; therefore,
282 hydrogen atoms were removed from the peptide prior to refining its conformation. The best peptide

conformation obtained from the FlexPepDock web server refinement was that with the lowest total energy score. The resulting peptide conformation was then applied the CHARMM36 force field using DV 4.0 to fix any errors in the structure of the peptide. Analysis of the interactions of DPP-IV with the peptide was performed using DV 4.0 by visualizing the final conformation of the peptide as two parts, as is sometimes done to infer protein-protein interactions (Verschueren, Vanhee, Rousseau, Schymkowitz, & Serrano, 2013).

2.12 Statistics

The software Statistica v. 7.0 (Statsoft, Tulsa, OK) was used to analyze mean values using analysis of variance (ANOVA). Differences were evaluated using the t-test with Tukey's adjustment. The significance level was set at p value < 0.05 .

3. Results and discussion

3.1 Soluble protein and antioxidant capacity

The total concentration of soluble protein (SP) from germinated and alcalase hydrolyzed common bean proteins, and their antioxidant capacity, are shown in **Table 1**. During the first 48 h of germination, the concentration of SP remained either constant or slightly decreased, being around 70%. After 72 h, an increase of SP could be observed reaching 80%. Germination of soybeans also increased the concentration of SP after 72 h (Vernaza et al., 2012). During germination, the proteins started to be degraded by endopeptidases, located in the cotyledons, forming oligopeptides. The oligopeptides are then hydrolyzed by exopeptidases in order to form free amino acids, which will be used to synthesize new proteins and tissues (Zakharov et al., 2004; Sangronis et al., 2006).

Alcalase hydrolysis increased the concentration of SP with time of reaction (**Table 1**). After 3 to 4 h of hydrolysis, the SP concentration slightly decreased, independently of germination time. Decreased concentration of SP after alcalase hydrolysis was also observed by Vernaza et al. (2012)

307 in soybean and by Rocha, Hernandez, Chang, and de Mejia (2014) in cowpea, which suggested a
308 similar behavior of alcalase action within these pulse proteins. During the hydrolysis procedure, it is
309 possible that very small peptides obtained after intense hydrolysis were not recovered, which may
310 cause the decrease in the SP determination.

311 The germination process considerably enhanced the antioxidant capacity after 24 h, from
312 136.90 to 505.59 $\mu\text{mol TE/g SP}$. The action of the endopeptidases on storage proteins during the
313 early stages of germination may have generated peptides with antioxidant properties. After 48 or 72
314 h of germination, the antioxidant capacity decreased, but was still higher than the no germinated
315 common bean. The longer the time of hydrolysis, the higher the antioxidant capacity was observed,
316 independently of germination time. Values around 1000 $\mu\text{M TE/g}$ of SP were obtained for the 4 h
317 hydrolyzed samples. Samples obtained after 3 or 4 h of hydrolysis had a higher concentration of
318 small peptides and lower SP value, which gave a higher antioxidant capacity if expressed as μmol
319 TE/g of SP in comparison to data expressed as $\mu\text{mol TE/g}$ dry hydrolyzed sample (**Table 1**).
320 Germination process and alcalase hydrolysis of common bean proteins positively influenced the
321 produced bioactive peptides with potent antioxidant properties capable to inhibit oxidative
322 degeneration caused by peroxy radicals, which triggers several diseases including cardiovascular
323 diseases and diabetes (Sarmadi & Ismail, 2010).

324 *3.2 Proteases and α -amylase inhibitors*

325 Some compounds considered as anti-nutritional were monitored during germination and
326 alcalase hydrolysis (**Table 2**). Bowman Birk and Kunitz inhibitors are trypsin/chymotrypsin
327 inhibitors, which reduce the breakdown of proteins during digestion; α -amylase inhibitor reduces the
328 breakdown of carbohydrates during digestion. Several studies demonstrated that legume germination
329 is able to decrease the concentration of these anti-nutritional compounds (Aguilera et al., 2013;
330 Vidal-Valverde et al., 2002; Paucar-Menacho, Berhow, Gontijo-Mandarino, Chang, & de Mejia,
331 2010; Dia et al., 2012). The concentration of Bowman-Birk inhibitor in the non-germinated and non-

332 hydrolyzed sample (G0-0h) was 6.6 mg/g of SP. This value is in agreement with those found by
333 Mojica and de Mejia (2015) for different varieties of common bean. The conditions used during
334 germination of the common bean did not alter this value. Alcalase hydrolysis decreased the Bowman
335 Birk concentration, except for the samples germinated for 24 h, which remained constant.

336 The Kunitz inhibitor concentration of the non-germinated sample was 0.7 mg/g of SP. This
337 inhibitor is usually low or absent in *Phaseolus* beans (Sathe, 2002). The germination process was
338 able to reduce the concentration of Kunitz inhibitor in 45% after 48 h. Alcalase hydrolysis, on the
339 other hand, resulted in an apparent increase of this inhibitor concentration, independently of the
340 germination time. It is possible that the alcalase hydrolysis may have released protein fractions that
341 were recognized by the primary antibody.

342 Carbohydrases inhibitors play an important role in the digestion of starch and they are
343 considered as a strategy for the treatment of T2D and obesity, because they can reduce the peak of
344 postprandial glucose (Sales, Souza, Simeoni, Magalhães, & Silveira, 2012). Germination for 24 h
345 (G24-0h) increased the α -amylase inhibitor concentration by 44% when compared to the non-
346 germinated sample (G0-0h). After 48 h of germination, the amount of α -amylase inhibitor was the
347 same as the non-germinated sample. Alcalase hydrolysis reduced the amount of α -amylase inhibitor
348 mainly after 2 h of hydrolysis. This indicates that the action of this protease resulted in peptides with
349 no ability to inhibit α -amylase. These results show that germination for 24 h of common bean may be
350 a source of peptides acting as α -amylase inhibitors able to impair the degradation of starches by α -
351 amylases.

352 3.3 SDS-PAGE electrophoretic profile

353 **Figure 1B** shows the electrophoretic profile from the germinated and alcalase hydrolyzed
354 common bean proteins. In common beans, the main storage proteins are globulins as the 7S
355 phaseolin and the 11S legumin. The phaseolin is composed by polypeptide subunits of 40 to 54 kDa
356 (Montoya et al., 2008). Phaseolin, with a molecular mass of about 50 kDa, was identified (**Figure**

357 **1B**, a) for all non-hydrolyzed samples, except for the sample germinated for 24 h (G24-0h). After 24
358 h of germination, the phaseolin band exhibited a decrease in the intensity suggesting that this protein
359 was degraded during this period of germination (**Figure 1B**, b). During the early stages of
360 germination, endopeptidases degrade the storage proteins to provide amino acids necessary to
361 develop new tissues (Sangronis et al., 2006; López-Pedrouso, Alonso, & Zapata, 2014). On the other
362 hand, López-Pedrouso et al. (2014) studied the phosphorylation of phaseolin during germination,
363 which can originate drastic conformational changes in the protein. Protein phosphorylation is a
364 reversible reaction that can occur in a short period of time. It is also possible that these
365 conformational changes occurred after 24 h of germination, and it was reversed after 48 and 72 h
366 changing it back as it was before germination. The conformational changes occurred after 24 h of
367 germination could have influenced the alcalase hydrolysis susceptibility, since the SP concentration
368 of the bean germinated for 24 h was lower than the others, independently of the hydrolysis time
369 (**Table 1**). Based on López-Pedrouso et al. (2014), protein conformational changes may be the
370 reason that bean germinated for 24 h did not decrease the Bowman-Birk inhibitor with alcalase
371 hydrolysis (**Table 2**).

372 Phaseolin was rapidly degraded after 1 h of alcalase hydrolysis, however, a thin band could
373 still be observed and its intensity decreased with time of hydrolysis. Oseguera-Toledo et al. (2011)
374 also performed alcalase hydrolysis of common bean proteins and found that phaseolin, was slowly
375 hydrolyzed, until complete degradation after 3 h. Alcalase hydrolysis resulted in the breakdown of
376 the phaseolin structure for all bean samples, independently of germination time (**Figure 1B**, b)
377 starting right after 1 h of hydrolysis. It could be observed that low molecular mass peptides of 15, 10
378 and smaller than 10 kDa were formed due to the alcalase action (**Figure 1B**, c and d).

379 A simulated gastrointestinal digestion was performed and the polypeptides formed were
380 characterized by SDS-PAGE electrophoresis as shown in **Figure 1C**. A band of about 26 kDa
381 (**Figure 1C**, e) could be observed for all non-hydrolyzed bean samples, except for the ones

germinated for 24 h (G24-0h). This band could be the result of the action of pepsin and pancreatin on phaseolin. The 24 h germinated sample did not show this band probably due to the germination metabolism that may degrade phaseolin by endopeptidases or changed its conformation due to phosphorylation. All alcalase-hydrolyzed samples exhibited a double band of 17 kDa (**Figure 1C**, f). These bands may be originated from those of 25 and 20 kDa obtained after alcalase hydrolysis. The action of the enzymes during gastrointestinal digestion decreased their molecular masses.

3.3 Molecular mass profile and peptide sequence identification

The HPLC-MS-MS sequences of the peptides from the digested bean samples for the non-germinated (G0) and 48 h of germination (G48) are shown in **Table 3**. The molecular mass of the peptides varied from 574.3 to 1448.8 Da. These peptides had their possible biological activities investigated from *in silico* analysis. Angiotensin converting enzyme (ACE) inhibition and dipeptidyl peptidase-IV (DPP-IV) inhibition were the main biological activities predicted for these peptides. DPP-IV inhibitors are a novel target for the treatment of diabetes. DPP-IV inhibitors prolong glucagon-like peptide-1 (GLP-1) action, which is responsible for lowering blood glucose levels through the stimulation of insulin secretion (Pathak & Bridgeman, 2010; Singh, Jatwa, & Joshi, 2014). Among the peptides identified, the peptide with the sequence RGPLVNPDPKPFL was found in the gastrointestinal digested bean sample germinated for 48 h and alcalase-hydrolyzed for 1 h, and originated from phaseolin, which is the main storage protein in common bean. This is a potential bioactive peptide with possible ability to inhibit DPP-IV and it can be obtained in a larger amount from the common bean storage proteins, helping to reduce the risk of T2D. The IAC-UNA cultivar of common bean had 24% of total protein (data not shown) and the yield obtained after germination and alcalase hydrolysis was 10% of the total bean weight. The RGPLVNPDPKPFL peptide is a fraction of phaseolin protein, which, according to Montoya et al. (2010), accounts for 40 to 50% of total protein in common beans. A calculation of the total yield of this specific peptide gave

0.36% of the total bean weight. Despite the low yield, this peptide can be synthesized, which is an easier way to obtain it in large amounts for further validation *in vivo*.

3.4 DPP-IV inhibition

DPP-IV inhibitors are currently being used as a drug to manage T2D (Kim, Nian, Doudet, & McIntosh, 2008). The concentration of common bean hydrolysates needed to inhibit 50% (IC₅₀) of the DPP-IV activity is shown in **Figure 2** in mg of dry hydrolysate per mL (A) and in mg of SP per mL (B). The lower the IC₅₀ concentration, the more potent a sample is at inhibiting DPP-IV. In order to verify the action of the bioactive peptides after ingestion, all bean samples were evaluated after simulated gastrointestinal digestion. The mixture of peptides obtained from the non-germinated and non-hydrolyzed bean sample (G0-0h) had an IC₅₀ of 1.2 mg of SP/mL. Mojica, Chen and de Mejia (2014) evaluated the IC₅₀ of DPP-IV activity of the protein of several common bean varieties; the authors found the IC₅₀ ranging from 0.1 to 1.0 mg of protein/mL for raw beans, which shows a great variation in the ability of the proteins to inhibit DPP-IV in common bean varieties. This low concentration needed to inhibit 50% of DPP-IV activity suggested that this bean sample might be a promising agent that can be used in the design of functional foods acting on the regulation of blood sugar levels and prevention of T2D. The IC₅₀ may still be improved by HPLC fractionation in order to purify the peptides capable to inhibit DPP-IV (Harnedy, O’Keeffe, & FizGerald, 2015); these authors found that the IC₅₀ improved from 1.49 to 0.54. Germination did not affect the DPP-IV IC₅₀ values as can be seen in samples G0-0h, G24-0h and G48-0h. This indicates that germination of common bean was not able to improve their ability as DPP-IV inhibitors, but it was not negatively influenced. Alcalase hydrolysis did not improve the DPP-IV IC₅₀. However, enzymatic hydrolysis of proteins can produce peptides with biological activities (Sarmadi & Ismail, 2010). One of the main biological activities found *in silico* for the individual peptides identified after alcalase hydrolysis was DPP-IV inhibition (**Table 3**). In addition, alcalase may have degraded the bioactive peptides that showed ability to block DPP-IV activity in the non-hydrolyzed samples.

431 3.5 Insulin secretion by INS-1E cells

432 Reduced glucose-stimulated insulin secretion from pancreatic β -cells is thought to be one of
433 the major causes of T2D (Szewczyk, 2014). The effect of common bean hydrolysates on insulin
434 secretion by INS-1E β -cells is shown in **Figure 3**. The concentration of 2 mg/mL of the non-
435 germinated and non-hydrolyzed bean (G0-0h) was able to stimulate the insulin secretion, increasing
436 45.3% from the basal state (control, 0 mM of glucose). Germination of common bean did not
437 increase the insulin secretion, and the insulin secretion remained the same as G0-0h. The bean
438 samples that underwent alcalase hydrolysis did not alter the insulin secretion; it remained the same as
439 the basal state. These results are in agreement to those of the DPP-IV inhibition activity that neither
440 germination nor alcalase hydrolysis affected the DPP-IV inhibition (**Figure 2**). According to Singh,
441 Jatwa, and Joshi (2014) the secretion of insulin by β -cells is related to the action of GLP-1, which
442 increases the amount of insulin released from the β -cells even before blood glucose levels become
443 elevated. DPP-IV, on the other hand, rapidly inactivates GLP-1, decreasing the insulin secretion
444 (Green, Flatt, & Bailey, 2006). It is possible that the DPP-IV inhibitory activity of the non-
445 hydrolyzed samples, independent of germination time, was able to avoid the GLP-1 inactivation,
446 leading to an increase in the amount of insulin released by the β -cells. Patients that went pancreas
447 transplantation were treated with sitagliptin; it was observed that the time needed to insulin therapy
448 was prolonged suggesting a positive effect of this DPP-IV inhibitor (Ergin, Poggio, Krishnamurhi,
449 Jaber, & Hatipoglu, 2015). The results of our non-hydrolyzed samples point in the same direction
450 like this active ingredient.

451 3.6 Computational modeling for interaction of DPP-IV and a common bean peptide

452 As showed in **Table 3**, the peptide with the sequence RGPLVNPDPKPFL (**Figure 4**) was
453 found in the simulated gastrointestinal digested bean sample germinated for 48 h and alcalase-
454 hydrolyzed for 1h. This peptide was selected because it originated from a main storage protein in

455 common bean, phaseolin and the *in silico* analysis showed possible biological activity as DPP-IV
 456 inhibitor. The interactions that the best conformation of this peptide had with DPP-IV are shown in
 457 **Figure 5**. The peptide had interactions with 35 amino acids of DPP-IV in which 12 were electrostatic
 458 and 23 were van der Waals. The interactions of the first six amino acids of the peptide
 459 RGPLVNPDPKPFL with DPP-IV are shown in Figure **5A**, and the remaining seven amino acids in
 460 Figure **5B**. Figure **5C** shows the way the peptide interacted with the active site of DPP-IV (Jadav et
 461 al., 2012); the amino acids of the S1 pocket (SER630, ASN710, HIS740) labeled in orange, S2
 462 pocket (GLU205, GLU206) in purple, and S3 pocket (SER209, PHE357, ARG358) in red. A red
 463 surface around the active site of DPP-IV (Figure **5D**) shows that the peptide was able to dock to this
 464 site. Interactions with these three pockets of DPP-IV are a sign that a compound might be a good
 465 inhibitor of DPP-IV as sitagliptin, a pharmaceutical DPP-IV inhibitor (Nabeno et al., 2013; Bower,
 466 Hernandez, Berhow & de Mejia, 2014). Interactions with the S3 pocket of DPP-IV are said to
 467 increase the DPP-IV inhibitory effect of a compound (Nabeno, et al., 2013), which the peptide
 468 RGPLVNPDPKPFL does. The total energy score of the complex between DPP-IV and the peptide as
 469 determined by the FlexPepDock server was -1410.876 Rosetta energy units (REU). The interaction
 470 score of DPP-IV and the peptide was -23.908 REU. REU are units of energy calculated by programs
 471 that use Rosetta algorithms in which a more negative number refers to a more stable state or better
 472 interaction, but it is not a physical unit. Protein-peptide docking of the bean peptide
 473 RGPLVNPDPKPFL to DPP-IV showed that the interaction score of the peptide was much lower (-
 474 23.908 REU) than the range of interaction scores published before for cowpea bean peptides (-5.671
 475 to -15.319 REU) (Rocha et al., 2014). This indicates that the peptide RGPLVNPDPKPFL is more
 476 likely to interact with DPP-IV than other peptides which have been previously docked to DPP-IV,
 477 using a similar docking methodology. Given its strong interaction energy score and predicted
 478 interactions with DPP-IV's active site, the peptide RGPLVNPDPKPFL is predicted to be a good
 479 inhibitor of DPP-IV.

4 Conclusion

Germination of common bean and further enzyme hydrolysis of its proteins can generate bioactive peptides with high antioxidant capacity. After germination of the common bean for 24 h, bioactive peptides formed achieved high α -amylase inhibition; this property may block the digestion of starch, decreasing the intestinal absorption of glucose. Germination and alcalase hydrolysis did not improve DPP-IV inhibition capacity or insulin secretion by β -cells; however, after the simulated gastrointestinal digestion, non-germinated and non-hydrolyzed bean samples, showed high DPP-IV inhibitory capacity and was able to stimulate the secretion of insulin. Common bean germination after 48 h and alcalase hydrolysis for 1 h released the peptide RGPLVNPDPKPFL from the main common bean storage protein phaseolin, which was able to inhibit DPP-IV by interacting with its active site. The ingestion of common bean may provide bioactive peptides that can manage and reduce the risk of T2D.

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639

640 **Figure Captions**

641 **Figure 1: A.** Pictures of germinated common bean after 0, 24, 48 and 72 h (G0, G24, G48, and G72,
642 respectively). **B.** Electrophoretic profile of common bean proteins at different germination and
643 hydrolysis times. **C.** Electrophoretic profile of common bean hydrolysates, after simulated
644 gastrointestinal digestion, at different germination and hydrolysis times. Std indicates the molecular
645 masses of pure proteins. G0 = no germination, G24 = 24 h of germination, G48 = 48 h of
646 germination, and G72 = 72 h of germination; 0, 1, 2, 3 and 4 h different alcalase hydrolysis times; **a**,
647 indicates molecular mass of 50 kDa; **b**, indicates molecular mass of 20 kDa; **c**, indicates molecular
648 mass of 10-15 kDa; **d**, indicates molecular mass of < 10 kDa; **e**, indicates molecular mass of 26 kDa,
649 and **f**, indicates molecular mass of 17 kDa.

650

651 **Figure 2:** Effect of different germination and alcalase hydrolysis times on common bean protein
652 subjected to simulated gastrointestinal digestion on inhibition of DPP-IV (IC₅₀). **A.** Expressed in mg
653 of dry hydrolysate per mL (mg hydrolysate/mL). **B.** Expressed in mg of soluble protein per mL (mg
654 SP/mL). SP = soluble protein. G0 = no germination, G24 = 24 h of germination, and G48 = 48 h of
655 germination; 0, 1, and 2 h different alcalase hydrolysis times. Lower case letters of each germination
656 period show significant differences among the different hydrolysis times ($p < 0.05$). Capital letters
657 show significant differences among germination times for each one of the different hydrolysis times
658 ($p < 0.05$). IC₅₀ = Concentration needed to inhibit 50% of the enzyme activity.

659

660 **Figure 3:** Effect of different germination and alcalase hydrolysis times on common bean protein
661 subjected to simulated gastrointestinal digestion on insulin secretion (μ IU/mL) by INS-1E β -cells.
662 G0 = no germination, G24 = 24 h of germination, and G48 = 48 h of germination; 0, 1 and 2 h

different alcalase hydrolysis times. Different letters indicate significant differences among the samples ($p < 0.05$).

Figure 4: Amino acid sequence, primary structure and physicochemical characteristics of the selected peptide obtained after germination, alcalase hydrolysis and simulated gastrointestinal digestion of common bean proteins. Amino acid nomenclature: D, aspartic acid; F, phenylalanine; G, glycine; K, lysine; L, leucine; N, asparagine; P, proline; R, arginine; V, valine.

Figure 5: The interactions that peptide RGPLVNPDPKPFL had with DPP-IV are shown with the first six amino acids of the peptide on one panel **5A** and the other seven amino acids in panel **5B**. The first and last amino acids of each fragment of the peptide are labeled with the type and number placement in the full peptide. Electrostatic (Pink) and van der Waals (Green) interactions are labeled according to the amino acid in DPP-IV that had the interaction. Hydrogen bonds are indicated by dashed arrows to side-chain (Blue) or main-chain (Green) amino acids with their distance labeled in angstroms. π -Cation interactions are labeled in orange. Some DPP-IV amino acids interact with more than one amino acid of the peptide, hence why the two panels displaying specific interactions (**5A-5B**) might show the same amino acid. The way the peptide interacts with the active site of DPP-IV (**5C**) shows the amino acids of the S1 pocket (SER630, ASN710, HIS740) labeled in orange, S2 pocket (GLU205, GLU206) in purple, and S3 pocket (SER209, PHE357, ARG358) in red. A red surface around the active site of DPP-IV (**5D**) shows that the peptide was able to dock to the active site of DPP-IV. SER=serine; ASN=asparagine; HIS=histidine; GLU=glutamic acid; PHE=phenylalanine; ARG=arginine.

685 **Table 1**686 Soluble protein and antioxidant capacity of common bean concentrated proteins at different
687 germination and hydrolysis times.¹

Germination Hydrolysis					
	0 h	1 h	2 h	3 h	4 h
<i>Soluble protein (%)</i>					
G0	73.0 ± 3.56bC	79.44 ± 7.01 bB	82.08 ± 7.48 aA	76.73 ± 1.67 bBC	68.93 ± 4.32 bD
G24	69.86 ± 5.90 bcB	76.76 ± 1.54 cA	67.76 ± 2.29 dB	57.22 ± 3.80 dC	54.69 ± 5.36 cC
G48	68.11 ± 0.28 cB	74.23 ± 7.59 cA	73.96 ± 4.98 cA	72.47 ± 4.94 cA	68.38 ± 6.91 bB
G72	80.17 ± 2.50 aB	81.11 ± 6.93 aB	80.70 ± 2.09 bB	87.24 ± 3.96 aA	86.13 ± 6.87 aA
<i>Antioxidant capacity (μmol TE/g hydrolyzed sample)</i>					
G0	99.77 ± 8.61 dD	458.01 ± 2.20 bC	557.34 ± 2.18 aC	780.19 ± 9.11 aA	701.88 ± 6.54 aB
G24	352.74 ± 29.73 aD	515.28 ± 50.32 aA	424.54 ± 12.99 bC	459.60 ± 21.45 cBC	498.30 ± 10.58 cAB
G48	252.75 ± 18.68 cC	549.54 ± 10.87 aB	551.29 ± 5.73 aB	740.01 ± 54.97 aA	744.58 ± 55.27 aA
G72	308.06 ± 10.45 bC	529.28 ± 3.73 aB	570.56 ± 29.61 aAB	640.59 ± 44.12 bA	625.70 ± 58.86 bA
<i>Antioxidant capacity (μmol TE/g soluble protein)</i>					
G0	136.90 ± 10.45 cC	578.80 ± 38.22 bB	682.07 ± 50.64 abB	1018.45 ± 84.93 aA	1020.63 ± 89.27 aA
G24	505.59 ± 32.01 aD	670.65 ± 60.80 abC	627.37 ± 38.92 bC	803.91 ± 18.90 bB	938.36 ± 82.59 aA
G48	371.15 ± 28.67 bC	742.22 ± 39.16 aB	716.80 ± 60.84 aB	1020.90 ± 6.66 aA	1085.00 ± 58.66 aA
G72	378.31 ± 4.62 bC	686.38 ± 4.02 abB	706.77 ± 18.41 aAB	733.39 ± 40.20 bA	721.47 ± 71.85 bA

688 G0 = no germination, G24 = 24 h of germination, G48 = 48 h of germination and G72 = 72 h of germination; 0, 1, 2, 3 and 4 h
689 different hydrolysis times. Different upper case letters of each group in the same row show significant differences among samples at
690 the same germination time ($p < 0.05$). Different lower case letters of each group in the same column show significant differences
691 among samples at the same hydrolysis time ($p < 0.05$).
692

693

694

695 **Table 2**

696 Bowman-Birk (BBI), Kunitz (KTI) and α -amylase inhibitors found in protein concentrates from
 697 common bean after different germination and alcalase hydrolysis times.¹

Germination period	Hydrolysis time (h)	Bowman-Birk (mg BBI/g SP)	Kunitz (mg KTI/g SP)	α -amylase (% AC/mg SP)
G0	0	6.63 $\pm$ 0.08 bc	0.69 $\pm$ 0.10 d	21.39 $\pm$ 1.34 b
	1	6.02 $\pm$ 0.04 d	1.15 $\pm$ 0.30 bc	21.74 $\pm$ 1.66 b
	2	5.81 $\pm$ 0.04 d	2.76 $\pm$ 0.01 a	13.27 $\pm$ 0.11 c
G24	0	6.91 $\pm$ 0.02 ab	0.70 $\pm$ 0.13 d	30.88 $\pm$ 2.45 a
	1	6.65 $\pm$ 0.06 abc	0.93 $\pm$ 0.08 cd	9.47 $\pm$ 0.86 cd
	2	6.96 $\pm$ 0.04 a	1.34 $\pm$ 0.13 b	13.02 $\pm$ 0.71 c
G48	0	6.80 $\pm$ 0.17 ab	0.38 $\pm$ 0.02 e	19.52 $\pm$ 0.08 b
	1	6.48 $\pm$ 0.10 c	1.42 $\pm$ 0.17 b	19.51 $\pm$ 1.08 b
	2	6.47 $\pm$ 0.05 c	0.65 $\pm$ 0.04 d	7.61 0.32 d

698 ¹ G0 = no germination, G24 = 24 h of germination, G48 = 48 h of germination; 0, 1 h, and 2 h of
 699 alcalase hydrolysis. SP = soluble protein. AC = inhibition relative to acarbose. Values followed by
 700 different letters in the same column show significant differences among samples ($p < 0.05$). n. s.=
 701 not significant.

702

Table 3: Sequences of peptides found by LC-MS-MS¹, their potential biological activity and common bean protein source, after simulated gastrointestinal digestion of concentrated protein from germinated and alcalase hydrolyzed common bean.²

Hydrolysis time (h)	Molecular mass (Da)	Sequence	Possible biological activities ^b	Common bean protein source ^c
<i>No-Germinated (G0)</i>				
0	958.52	SAKFPPAGGK	ACE inhibitor, DPP-IV inhibitor, hypotensive	Monoglyceride lipase, ubiquitin conjugating enzyme 2
	961.56	KPTAPPAAPL	ACE inhibitor, antioxidative, DPP-IV inhibitor	Nitrite reductase, plasma membrane intrinsic protein 1
	980.54	NKGAAPPAAGK	ACE inhibitor, DPP-IV inhibitor	Nodule-enhanced protein phosphatase type 2C, upstream element-binding protein, lysine histidine transporter 1-like protein, RuBisCO large subunit-binding protein subunit α , dolichyl-diphosphooligosaccharide protein glycosyltransferase subunit STT3A
	1090.68	SGGYKLLLLK	ACE and DPP-IV inhibitor, stimulating, antioxidative	CNL-B29, polyprotein
1	643.4	TRGVLV	ACE inhibitor, stimulating	Cyclic nucleotide-gated channel C, starch synthase II precursor, α -galactosidase
	706.33	SKDGGPF	Antiamnestic, ACE inhibitor, antithrombotic, regulating, antioxidative, DPP-IV inhibitor	Auxin-induced protein, mitochondrial 2-oxoglutarate/malate carrier protein, phototropin, ascorbate peroxidase, O-acyltransferase WSD1, H(+)-transporting ATPase, gag-pol polyprotein, urease accessory protein ureG, subtilis in-like protease 1, tonoplast intrinsic protein β , chlorophyll a/b-binding protein, chlorophyll a/b-binding protein, aquaporin TIP-typeprotein
	980.57	KPGSGLPAAAL	Antiamnestic, ACE inhibitor, antithrombotic, regulating, antioxidative, DPP-IV inhibitor	Vacuolar amino acid transporter 1
	1138.68	LVNLVPPYPK	ACE inhibitor, stimulating, DPP-IV inhibitor	Tubulin α -3 chain-like protein, tubulin A
<i>Germinated 48 h (G48)</i>				
0	738.39	KGAGGRGH	ACE inhibitor	E3 ubiquitin-protein ligase HERC1-like protein, senescence-associated protein, bidirectional sugar transporter SWEET12, oxygen-evolving enhancer protein 1, TIFY protein, endo-13;14- β -D-glucanase-like protein
	958.55	SLNLWLTL	ACE inhibitor	bZIP transcription factor 2, coronatine-insensitive protein 1-like protein, TOC1, xyloglucan endotransglucosylase/hydrolase protein 9
1	574.32	KTGGGR	ACE inhibitor	14-3-3 protein, target of Myb protein 1-like protein, chlorophyll a/b-binding protein (LHCII type II), Thaumatin-like protein, ascorbate peroxidase
	728.36	TTGSR AH	ACE inhibitor, antioxidative	Lipid binding protein, cytosolic glutathione reductase, NPH3 family protein
	980.55	RGPAAPLGSR	Antiamnestic, ACE inhibitor, antithrombotic, opioid, regulating, DPP-IV inhibitor	ADP-glucose pyrophosphorylase small subunit PvAGPS1, alcohol dehydrogenase 1
	1252.65	LLYEVELVFE	ACE inhibitor, stimulating, antioxidative, DPP-IV inhibitor	Alanine glyoxylate aminotransferase, serine glyoxylate aminotransferase 2, nerol synthase
	1448.81	RGPLVNPD PKPFL	Antiamnestic, ACE inhibitor, antithrombotic, stimulating, regulating, antioxidative, DPP-IV	Phaseolin, α -phaseolin, α -galactosidase-like protein

¹ Peptides obtained from the HPLC elution profile with intensity of at least 50%; ² G0 = no germination, G48 = 48 h of germination; 0, and 1h of alcalase hydrolysis; Amino acid nomenclature: C, cysteine; H, histidine; I, isoleucine; M, methionine; S, serine; V, valine; A, alanine; G, glycine; L, leucine; P, proline; T, threonine; F, phenylalanine; R, arginine; Y, tyrosine; W, tryptophan; D, aspartic acid; N, asparagine; E, glutamic acid, Q, glutamine; K, lysine; ^b Determined using BIOPEP; ^c Determined using BLAST tool.

Figure 1

Figure 2

Figure 3

Figure 4

RGPLVNPDPKPFL

Theoretical physicochemical characteristics

Sequence	Length	Molecular mass (Da)	Isoelectric point	Net charge	Hydrophobicity (kcal/mol)
RGPLVNPDPKPFL	13	1448.81	10.14	+1	+14.04

Figure 5