Identification of ACE-inhibitory peptides from *Phaseolus vulgaris* after *in vitro* gastro-intestinal digestion

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Abstract

The objective of the present study was to identify the ACE-inhibitory peptides released from thermally treated *Phaseolus vulgaris* (pinto) whole beans after *in vitro* gastro-intestinal digestion. The degree of hydrolysis increased during digestion reaching a value of 50% at the end of the pancreatic digestion. The < 3 kDa fraction of the post-pancreatic sample showed high ACE-inhibitory activity (IC_{50} = 105.6 ± 2.1 µg of peptides/mL). Peptides responsible for the ACE-inhibitory activity were isolated by reverse phase HPLC. Three fractions, showing the highest inhibitory activity were selected for MS/MS experiments. Eleven of the identified sequences have previously been described as ACE-inhibitors. Most of the identified bioactive peptides had a hydrophobic amino acid, (iso)leucine or phenylalanine, or proline at the C-terminal position, which is crucial for their ACE-inhibitory activity. The sequence of some peptides allowed us to anticipate the presence of ACE-inhibitory activity.

Keywords: common bean; proteolysis; hypertension; mass spectrometry; peptides

Introduction

High blood pressure or hypertension is a major independent risk factor for cardiovascular and related diseases. Inhibition of the angiotensin I-converting enzyme (ACE) is a key point in the treatment of hypertension. ACE is a dipeptidyl carboxypeptidase that catalyses, *in vivo*, the conversion of the plasmatic peptide angiotensin I into the potent vasoconstrictor angiotensin II. Moreover, ACE can also degrade the antihypertensive vasodilator bradykinin. Inhibition of ACE plays an important role in regulation of blood pressure and drugs that inhibit ACE are commonly prescribed for the treatment of hypertension or related cardiovascular diseases (Acharya et al., 2003).

Bioactive peptides derived from parent proteins, either from animal or plant sources, are thought to be beneficial in the management of hypertension, because of their ability to inhibit the activity of the enzyme ACE (Phelan, & Kerins, 2011; García et al., 2013).

Many ACE-inhibitory peptides have been isolated from milk or its derivatives through processes of enzymatic hydrolysis, fermentation and *in vitro* gastrointestinal digestion (Phelan, & Kerins, 2011). In addition to milk proteins, biologically active and ACE-inhibitory peptides can be released from plant derived food proteins following enzymatic hydrolysis. Cereals and legumes are the main target of these researches, both being rich sources of proteins with a complementary spectrum of amino acids (García et al., 2013). ACE-inhibitory peptides have been identified after hydrolysis by commercial enzymes (such as alcalase) of mungbean, chickpea, rice, soybean and corn gluten meal (Garcia et al., 2013). Another process commonly used to generate ACE-inhibitory peptides is the *in vitro* sequential gastro-intestinal digestion. Regarding vegetables, this approach has been used to release ACE-inhibitory peptides from spinach, buckwheat and sunflower (Garcia et al., 2013).

Common bean (*Phaseolus vulgaris*) is, together with soybean and peanuts, one of the most important food legumes in the world. It contain high levels of protein (20-30% on a dry basis),

40 fiber, as well as valuable minerals and vitamins. The most important proteins found in common
41 bean are the storage protein phaseolin (40– 50% of the total) and lectins (10–27% of the total).
42 Various epidemiological and clinical studies, suggested an association between the consumption of
43 common bean and legumes and the incidence of chronic diseases (Luna-Vital et al., 2014 and
44 references herein). For instance, the NHANES I epidemiologic follow-up study indicated that the
45 consumption of more than 4 serving of legumes (beans and peas) per week was correlated to a
46 reduction in the cardiovascular and coronary heart disease as well as in the development of
47 hypertension in US men and women (Bazzano et al., 2001).
48 Recently, some studies demonstrated that hydrolysis of proteins isolated from several varieties of
49 *Phaseolus vulgaris* lead to the release of ACE-inhibitory compounds (Torruco-Uco et al., 2009; Rui
50 et al., 2012a; Rui et al., 2012b; Boschin et al., 2014; Mojica et al., 2015). These studies suggested
51 that ACE-inhibition by bean-derived peptides may be a mechanism of action explaining the
52 epidemiological evidences related to a lower incidence of hypertension and cardiovascular disease
53 in humans. However, in only one study the peptides responsible for the ACE-inhibitory activity was
54 identified (Rui et al., 2013).
55 To expand this knowledge, the current study was undertaken to identify the peptides responsible for
56 the ACE-inhibitory activity released from common bean after gastro-intestinal hydrolysis.
57 We used an *in vitro* digestion procedure mimicking the chemical and physical condition of the
58 gastro-intestinal tract to process thermal-treated pinto bean (*Phaseolus vulgaris*). The digested
59 fraction was characterized for the ACE-inhibitory activity and then further separated with HPLC.
60 Finally, the different fractions containing low molecular weight peptides were characterized for
61 their ACE-inhibitory activity. The fractions with the highest activity were then analyzed with
62 nanoLC-QTOF-MS with the aim to identify the bioactive peptides.

63 **Methods**

64

65 **Materials**

66 Bile salts (mixture of sodium cholate and sodium deoxycholate), porcine α-amylase, pepsin from
67 porcine gastric mucosa, pancreatin from porcine pancreas (4xUSP), angiotensin converting enzyme
68 from rabbit lung, mucin II and III, bovine serum albumin, lysozyme and urea were supplied by
69 Sigma (Milan, Italy). Amicon Ultra-4 regenerated cellulose 3 kDa were supplied by Millipore
70 (Milan, Italy). *Phaseolus vulgaris* beans (pinto beans) were purchased from a local market (Reggio
71 Emilia). All electrophoretic, HPLC and MS/MS reagents were from Biorad (Hercules CA, U.S.A.).
72 All the other reagents were from Carlo Erba (Milan, Italy). The absorbance was read using a Jasco
73 V-550 UV/Vis spectrophotometer (Orlando FL, U.S.A.).

74

75 **Sample preparation**

76 Pinto beans were prepared by cooking 200 g of beans in 1000 mL of boiling water for 2h. Cooked
77 pinto beans were brought to room temperature and then subjected to the *in vitro* digestion protocol.

78

79 **In vitro gastro-intestinal digestion**

80 For the *in vitro* digestion, the protocol, developed within the COST Action FA1005 and further
81 validated for milk and solid food (Kopf-Bolanz et al., 2012; Stuknite et al., 2014), was followed.
82 Simulated salivary (SSF), simulated gastric (SGF), and simulated intestinal (SIF) fluids were
83 prepared according to Kopf-Bolanz et al. (2012). Intestinal fluid was prepared by mixing pancreatic
84 (PF) and bile (BF) fluids.
85 *Phaseolus vulgaris* beans (15.75 g) were homogenized in a stomacher laboratory blender for 1 min
86 to simulate mastication in the presence of 21 mL of SSF and further incubated for 5 min (oral
87 phase). Porcine α-amylase was added to SSF (150 U/mL of SSF) immediately before the
88 incubation. Afterwards, 42 mL of SGF was added, the pH was adjusted to 2.0 with HCl and

89 supplemented with porcine pepsin (315 U/mL of SGF). The samples was further incubated for 120
90 min (gastric phase). The intestinal digestion was carried out by adding to the gastric digested beans
91 63 mL of SIF (42 mL of PF and 21 mL of BF), adjusting the pH to 7.0 and incubating the sample
92 for 120 min (pancreatic phase). Pancreatin was added to the PF immediately before the incubation.
93 All incubations were performed at 37°C on a rotating wheel (10 rpm).
94 Control digestions were carried out by replacing pinto beans with the same amount of water.
95 Aliquots of the digested samples were collected at the end of each phase, cooled in ice and
96 immediately frozen at –80 °C for further analysis.
97 The digestions were performed in triplicate.
98
99 **Determination of the degree of hydrolysis (DH)**
100 The determination of protein hydrolysis in the digested samples was carried out by measuring the
101 peptide concentration using the TNBS method with leucine as standard (Adler-Nissen, 1979).
102 The hydrolysis degree was calculated as reported in equation (1):
103 $\mathbf{DH = (h/h_{tot}) \cdot 100}$ (1)
104 where **h** is the hydrolysis equivalent, defined as the concentration in milliequivalents/g of protein of
105 α-amino groups formed at the different stages of the simulated digestion, and $\mathbf{h_{tot}}$ is the hydrolysis
106 equivalent at complete hydrolysis to amino acids. The total number of amino groups was
107 determined by hydrolyzing the total protein extract in 6 mol/L HCl at 110°C for 24 h. The $\mathbf{h_{tot}}$ value
108 was calculated resulting in 7.43 milliequivalents/g of protein.
109 The total protein extract from pinto beans whole seeds was prepared according to Carrasco-Castilla
110 et al. (2012).
111
112 **SDS-PAGE electrophoresis**
113 Samples taken at different times of digestion were subjected to SDS-PAGE electrophoresis using
114 13% polyacrylamide separating gel according to Carrasco-Castilla et al. (2012). Samples were

115 diluted to similar end dilutions in Laemmli buffer (0.05 mol/L Tris, pH 6.8, containing 2% SDS, 0.1
116 mol/L DTT, and 0.025% Bromophenol Blue). Vials were heated in boiling water for 4min, and 10
117 µL of each sample (corresponding to 20 µg of undigested pinto bean proteins) was applied to the
118 gel. As a molecular ladder, the Blue-Step^{TM} Broad range marker (14–200 kDa) was used. Gels were
119 stained with Coomassie Blue.
120

121 **Measurements of ACE-inhibitory activity**

122 Samples (4 mL) collected at the end of the pancreatic step of the *in vitro* digestion were subjected to
123 ultrafiltration with Amicon Ultra-4 nominal cut-off 3kDa, at 7500*g* for 120 min at 4°C. The filtrates
124 containing low molecular weight peptides was further analyzed for their ability to inhibit ACE
125 activity. Peptides were quantified in the sample by using the TNBS method as described in
126 paragraph 2.4. Results are expressed as mg of leucine equivalent/g of pinto bean.
127 ACE-inhibitory activity was measured by the spectrophotometric assay of Ronca-Testoni (1983)
128 using the tripeptide, 2-furanacryloyl-phenylalanylglycylglycine (FAPGG) as substrate.
129 For the control assay, 350 µL of FAPGG 1.6 mM dissolved in the reaction buffer (Tris-Cl 100
130 mmol/L pH 8.2 and containing 0.6 mol/L of NaCl) was mixed directly in cuvette with 330 µL of
131 reaction buffer. The solution was kept at 37°C for 5 min before the addition of 20 µL of ACE
132 solution (so that the final activity of the enzyme in the assay was 50 mU/mL).
133 For the inhibition assay, variable amount of sample were added in place of the buffer.
134 The reaction was followed at 345 nm for 10 min.
135 Results are expressed as IC_{50} that is defined as the concentration of peptides required to inhibit 50%
136 of the enzymatic activity.
137

138 **HPLC analysis of peptides**

139 HPLC separation of the low molecular weight fractions of digested pinto beans was performed with
140 a Jasco HPLC system equipped with a reversed phase column Hamilton HxSil C18 (Hamilton,

141 Reno, Nevada; 250mm x 4.6mm) as described in Hernández-Ledesma et al. (2007). The two
142 solvents were: solvent A mixture of water-trifluoroacetic acid (0.037%) and solvent B acetonitrile-
143 trifluoroacetic acid (0.027%). A linear gradient of solvent B in A ranging from 0% to 45% in 115
144 min with a flow rate of 0.5 mL/min was used to separate the peptides contained in the low
145 molecular fractions of digested milk. The PDA detector was set at 214 nm. Five major fractions
146 were collected and freeze-dried. These fractions were characterized for their ACE-inhibitory
147 activity (paragraph 2.6) and for the peptide concentration (paragraph 2.4).
148
149 **Nanoflow LC-ESI-QTOF-MS/MS analysis**
150 The fractions with the highest ACE-inhibitory activity were subjected to QTOF MS/MS analysis for
151 peptides identification. Nano LC/MS and tandem MS experiments were performed on a 1200 Series
152 Liquid Chromatographic two-dimensional system coupled with a 6520 Accurate-Mass Q-TOF
153 LC/MS via a Chip Cube Interface (Agilent Technologies). Chromatographic separation was
154 performed on a ProtID-Chip-43(II) including a 4 mm 40 nL enrichment column and a 43 mm × 75
155 µm analytical column, both packed with a Zorbax 300SB 5 µm C18 phase (Agilent Technologies).
156 The mobile phase consisted of (A) H_2O/acetonitrile/formic acid (96.9:3:0.1, v/v/v) and (B)
157 acetonitrile/H_2O/formic acid (94.9:5:0.1, v/v/v). The sample (4 µL) was loaded on the Chip
158 enrichment column at a flow rate of 4 µL/min with a mobile phase consisting of 100% A using a
159 G1376A capillary pump. A flush volume of 2 µL and a flush-out factor of 2 were used. After valve
160 switching a gradient elution was performed with the enrichment and analytical column at 500
161 nL/min using a G2226A nano pump. The gradient started at 3% B for 0.5 min then linearly ramped
162 up to 28% B in 16.5 min. The mobile phase composition was raised up to 40% B in 3 min, then
163 95% B in 1 min and maintained for 4 min in order to wash both enrichment and analytical columns.
164 The mass spectrometer was tuned, calibrated and set with the same parameters as reported by Dei
165 Più et al. (2014). For identification, MS/MS spectra were converted to .mgf and de novo peptide
166 sequencing was performed using Pepnovo software. The following parameters were considered:

167 enzyme, none; peptide mass tolerance, ± 40 ppm; fragment mass tolerance, ± 0.12 Da; variable
168 modification, oxidation (M) and phosphorylation (ST); maximal number of PTMs permitted in a
169 single peptide 3.
170 A search for the biological activity of peptides identified was carried out through the BIOPEP
171 database (http://www.uwm.edu.pl/biochemia/biopep/start_biopep.php). Confirmation of peptides
172 sequence in common bean proteins was performed using Peptide Match
173 (http://research.bioinformatics.udel.edu/peptidematch/index.jsp).
174
175 **Statistical analysis**
176 All data are presented as mean ± SD for three replicates for each prepared sample. Univariate
177 analysis of variance (ANOVA) with Tukey post-hoc test was applied using Graph Pad Prism 6.0
178 (GraphPad Software, San Diego, CA). The differences were considered significant with $P < 0.05$.

179 **Results**

180

181 **Assessment of protein hydrolysis during simulated digestion**

182 Cooked pinto beans (*Phaseolus vulgaris*) were submitted to a consecutive three-step *in vitro* gastro-
183 intestinal digestion protocol. Protein hydrolysis during the digestion was followed both by
184 measuring the amount of released free amino group (degree of hydrolysis) and by SDS
185 electrophoresis.

186 The degree of hydrolysis increased after peptic digestion although not significantly (**Figure 1**). The
187 pancreatic digestion produced a high and significant increase ($P<0.001$) in protein hydrolysis
188 reaching a degree of hydrolysis value of 50%.

189 SDS–PAGE analysis of the total bean protein preparation and of the different steps of digestion is
190 shown in **Figure 2**. Several bands were present in the total bean protein preparation (**Figure 2; lane**
191 **2**). The most abundant bands were the phaseolins subunits α and β with a molecular weight (MW)
192 of 47 and 44 kDa (Montoya et al., 2008). Bands between 16 and 32 kDa corresponded to proteins
193 belonging to the family of lectins. Other bands were visible such as that at 10 kDa which
194 corresponded to protease inhibitor, and bands at 15.2 and 33.8 kDa corresponding to α-amylase
195 inhibitor and its β-subunits (Carrasco-Castilla et al., 2012). After the oral phase of the digestion
196 (**Figure 2; lane 3**) only the band corresponding to the subunit β of phaseolin was visible, together
197 with some bands at lower MW which corresponded to lectin family proteins. Gastric digestion
198 resulted in the disappearance of all the visible bands suggesting that the major proteins extracted
199 during the oral phase were degraded during this phase (**Figure 2; lane 4**). Similarly after pancreatic
200 digestion (**Figure 2; lane 6**) no bands corresponding to the bean proteins were found. The bands in
201 lane 6 corresponded to the digestive pancreatic enzymes since they showed the same electrophoretic
202 profile as the control digestion carried out without beans (**Figure 2; lane 7**).

203

204 **ACE-inhibitory activity**

205 The ACE-inhibitory activity of the < 3 kDa permeates was calculated as IC_{50}. While the < 3 kDa
206 fraction of undigested sample showed no effect, the ACE-inhibitory activity at the end of the
207 gastro-intestinal was determined to be 105.6 ± 2.1 µg of peptides/mL.
208 The permeate < 3 kDa fraction was loaded on the HPLC C18 column and peptides detected at 214
209 nm with a photodiode array detector. The chromatogram presented in **Figure 3** showed most
210 peptides eluting between 6 and 25 min. As shown in **Figure 3**, five fractions (F1–F5) were
211 collected. All fractions exerted some ACE-inhibitory activity. **Table 1** shows the ACE-inhibitory
212 activity, expressed as IC_{50} values, of the peptide fractions isolated by RP-HPLC. IC_{50} values ranged
213 from 5.4 to 18.1 µg/mL. In fractions F1, F3 and F4 a higher ACE inhibitory activity was observed.
214 The lowest IC_{50} values of 5.4 ± 0.2 and 5.5 ± 0.1 µg peptides/mL were found in fractions F4 and
215 F3, respectively, which were about twenty times lower than the IC_{50} value of the post-pancreatic <
216 3 kDa permeate.
217 The yield of the five collected fractions was estimated (**Table 1**). For fractions F2, F3, F4 and F5
218 the yield was similar, ranging from 12.4 and 14.8%. Fraction F2 showed the highest yield of
219 approximately 22% (**Table 1**). The sum of the peptide concentration in the five fractions resulted in
220 3.42 mg/mL, giving a total yield of 75.5%.
221
222 **NanoLC-ESI-QTOF-MS/MS identification of peptides in the HPLC collected fractions**
223
224 Fractions with the highest ACE-inhibitory activity were then analyzed with nanoflow LC-ESI-
225 QTOF mass spectrometry to identify the peptides present in these fractions.
226 Each peak was selected for peptide identification by MS/MS ion scan using de novo sequencing
227 software. Results from peptide identification were subjected to a manual evaluation, and the
228 validated peptide sequences explained most of the major peaks in the MS spectra. The list of
229 compounds identified in these fractions is shown in **Tables 2-4**. In addition, the list of peptides
230 identified in the fraction F2 and F5 is shown as online supplementary material (**Tables S1** and **S2**).

231 Fraction F1 (**Table 2**) contained 10 peptides, most of them being dipeptides (7 peptides; 70% of the
232 identified peptides in F1). The longest peptide identified in this fraction (EEEES) had five amino
233 acid residues and derived from the α and β subunits of phaseolin. Fraction F2 (**Table S1**) showed a
234 45% of dipeptides (4 out of 9) with the longest peptides having five amino acid residues. In fraction
235 F3 (**Table 3**), 13 peptides, having 2-5 amino acid residues, were identified. Five peptides (~ 38% of
236 the identified peptides in F3) were dipeptides. The range of peptide length in F4 (**Table 4**) was
237 between 2 and 7 amino acid residues. Eight peptides from 24 are dipeptides (~ 33% of the identified
238 peptides in F4) as well as eight peptides contained three amino acid residues. The longest peptide
239 found in this fraction was the eptapeptide SGSGDEV derived from the α and β subunits of
240 phaseolin. Fraction F5 (**Table S2**) contained the lowest percentage of dipeptides (18%).
241 In general, the length of peptides increased with increasing acetonitrile concentration. The average
242 number of amino acid residues per peptide (sum of total amino acid residues in the fraction/number
243 of peptide in the fraction) in the five fractions increased from 2.5 in F1 to 2.8 in F2, 3.1 in F3, 3.4 in
244 F4 and 4.3 in F5.
245 As can be seen in **Tables 2-4**, each fraction contained at least two peptides with previously reported
246 ACE-inhibitory activity.
247 Finally, some free amino acids (not reported in the tables) were found in three of the characterized
248 fractions. In F1 we identified methionine (M), glutamine (Q) and valine (V) whereas in F3 the
249 identified amino acids were threonine (T) and tyrosine (Y) and in F4 leucine/isoleucine (Lx).

250 **Discussion**

251

252 To our knowledge, the present study is the first demonstration that ACE-inhibitory peptides can be
253 released during the gastro-intestinal digestion of whole pinto beans.
254 Previous researches (Mojica et al., 2015; Rui et al. 2012a and 2012b; Boschin et al., 2014) showed
255 the potential of bean proteins to release ACE-inhibitory peptides during hydrolysis catalyzed by
256 bacterial or gastro-intestinal enzymes. However, these works focused on the bioactive properties of
257 peptides released from protein isolates from beans and not from whole beans.
258 The proteolysis process during *in vitro* gastrointestinal digestion can be strongly influenced by food
259 matrix, e.g. by reducing the proteolysis rate of proteins (Mandalari et al., 2011). However, in most
260 studies performed so far, pure proteins, extracted from whole bean, have been subjected to
261 enzymatic digestion assays excluding a potential impact of a food matrix. Common bean is a
262 complex matrix containing not only proteins but also polysaccharides and polyphenols which can
263 have a strong impact on gastrointestinal degradation of protein. For example, polysaccharides were
264 shown to hamper pepsin activity (Polovic et al., 2007) whereas phenolic compounds may have a
265 positive or a negative effect on protein digestion depending on the protease and on the nature of the
266 phenolic compound (Tagliazucchi et al., 2005). Thus, to give a picture more closely to the *in vivo*
267 physiological condition of the bioactive peptides released during *in vitro* digestion, food matrix
268 should be considered.
269 The degree of hydrolysis, which represents the extent of the digestion, increased reaching a value of
270 50% at the end of the pancreatic digestion. This value is comparable to that obtained by Mojica et
271 al. (2015) after simulated digestion of precooked pinto beans protein extract.
272 Previous researchers have reported a lower degree of hydrolysis after digestion of bean protein
273 extracts. Rui et al. (2012a), obtained a degree of hydrolysis of 16% after simulated digestion of
274 pinto bean protein extracts non-thermally treated. They digested nine varieties of beans achieving a
275 degree of hydrolysis between 7 and 16%. Pepsin-pancreatin digestion of hard-to-cook bean

276 extracted proteins resulted in a degree of hydrolysis of 28% (Betancour-Ancona et al., 2014). In
277 general, bean protein digestibility in a simulated digestive system seems to be related to the thermal
278 treatment. Cooking of beans resulted in a higher degree of hydrolysis respect to non-cooked beans.
279 Bean proteins, especially phaseolin, are considered highly resistant to enzymatic hydrolysis
280 (Jivotovskaya et al., 1996). However, thermal processing may result in a loss of the tri-dimensional
281 structure of the proteins, causing unfolding of the molecules. Thermally denatured bean proteins are
282 more susceptible to enzymatic hydrolysis (Montoya et al., 2008; Rui et al., 2012b)**.** Protein
283 unfolding results in an increased exposure of the cleavage sites, facilitating the access to these sites
284 of digestive enzymes that are able, with greater efficiency, to hydrolyze proteins.
285 The extent of the hydrolysis also depend on the type of protease used. Despite pepsin-pancreatin
286 digestion, without thermal processing, results in low (< 30%) degree of hydrolysis (Rui et al.,
287 2012a; Betancour-Ancona et al., 2014), treatment with alcalase, termolysine, flavourzyme or a
288 combination of these resulted in a high degree of hydrolysis (35-70%) (Torruco-Uco et al., 2009;
289 Rui et al., 2012b; Valdez-Ortiz et al., 2012).
290 Protein digestibility and the extent of hydrolysis affected the *in vitro* ACE-inhibitory activity.
291 Generally speaking, a higher degree of enzymatic hydrolysis represents a higher ACE-inhibitory
292 activity. Digested pinto beans released ACE-inhibitory peptides with a calculated IC_{50} value in the
293 post-pancreatic < 3 kDa permeate of 105.59 µg of peptides/mL. This value is about two times
294 lower than the IC_{50} found by Mojica et al. (2015) and Rui et al. (2014) after simulated digestion of
295 pre-cooked pinto beans and a protein extract from pinto beans, respectively. This difference can
296 arise from the different digestion system used and/or from the ultrafiltration step. The digestion
297 system used in our study has been validated for liquid and solid foods (Kopf-Bolanz et al., 2012;
298 Stuknite et al., 2014) and developed within the COST Action FA1005.
299 This *in vitro* digestion system closely mimics the digestive process in humans, especially for protein
300 digestion, resulting in the formation of free amino acids and small peptides (2-6 amino acids)
301 (Kopf-Bolanz et al., 2012). Data reported in **Tables 1-4** and **Tables S1** and **S2** are in agreement

302 with the results of the Kopf-Bolanz study. The five collected fractions explained the 76% of the
303 total peptides present in the digested bean sample. The identification of the peptides in these
304 fractions showed that the length of the peptides was between 2 and 7 amino acid residues. We
305 identified in the five fractions a total of 67 peptides. About 39% of the peptides are dipeptides (26
306 dipeptides), whereas tripeptides represent the 28% of the total identified peptides. The average size
307 of the identified peptides was 3.3 residues per peptides. To this purpose, it has been suggested that
308 short peptides (2-12 amino acid residues) are more effective inhibitors of ACE activity than longer
309 peptides (Garcia et al., 2013).
310 Furthermore, ultrafiltration is a technique largely used to enrich the digested food in ACE-
311 inhibitory peptides. For example, Vermeirssen et al. (2005), found higher ACE-inhibitory activity
312 in the < 3 kDa fraction than in the > 3 kDa fraction and in un-fractionated digested pea and whey
313 samples.
314 Among the identified peptides, several had previously been established to display ACE-inhibitory
315 activity. More specifically, two dipeptides in fraction F3 showed very low IC_{50} values. The
316 dipeptides EY and AI, which were previously isolated from shark meat hydrolysate and soy sauce-
317 like seasoning, demonstrated IC_{50} values of 2.7 and 3.4 µmol/L, respectively (Wu et al., 2008;
318 Nakahara et al., 2010). The presence of these two small peptides justifies the high ACE-inhibitory
319 activity found in this HPLC fraction. Fraction F4, instead, contains two tripeptides with high ACE-
320 inhibitory activity. The tripeptide LAP (isolated from hydrolyzed chicken muscle) and its isomeric
321 form IAP (isolated from wheat gliadin hydrolyzates) are potent inhibitors of ACE activity with IC_{50}
322 values of 3.5 and 2.7 µmol/L, respectively (Fujita et al., 2000; Motoi & Kodama, 2003). LAP was
323 found to be effective when intravenously administered to spontaneously hypertensive rats (SHR)
324 (Fujita et al. 2000). Also the tripeptide LVE, previously isolated from pearl oysters, showed low
325 IC_{50} value of 14.2 µmol/L (Qian et al., 2007). Additional dipeptides with higher IC_{50} values were
326 found in fraction F3 (GLx) and F4 (PL, GF, SF and AF) as reported in **Tables 3** and **4**. Fraction F1,

327 instead, contained dipeptides (LF, IF and EV) with previously demonstrated ACE-inhibitory
328 activity which showed IC_{50} values ranged between 350-930 µmol/L (see **Table 2**).
329 According to previous reports on the structure–activity correlations between different peptide
330 inhibitors of ACE (Wu et al., 2006), some other peptides may have the potential to inhibit ACE
331 activity. In the case of the dipeptides, for both positions, amino acid residues with large bulk chain
332 as well as hydrophobic side chains such as aromatic amino acids (phenylalanine, tyrosine and
333 tryptophan) and branched aliphatic side amino acids (leucine, isoleucine and valine) are preferred
334 (Wu et al., 2006). Based on these considerations, three dipeptides in fraction F4 (VLx, ELx and DF)
335 and two dipeptides in fraction F3 (DLx and TLx) could be active against ACE activity. All these
336 peptides have a hydrophobic amino acid residue, L or F, at the C-terminal position, showing they
337 display structural characteristics of ACE-inhibitory peptides.
338 To exert an antihypertensive effect after oral ingestion, ACE-inhibitory peptides have to be
339 adsorbed at intestinal level and arrive at the cardiovascular system in an active form. Peptides
340 identified in our study are very short (di- or tri-peptides) and resistant to gastro-intestinal digestion
341 by pancreatic proteases such as trypsin, chymotrypsin, elastase and carboxypeptidases. All these
342 proteolytic enzymes are present in our digestive system and are responsible for the production of
343 these ACE-inhibitory peptides.
344 Exopeptidases in the brush-border membrane may limit the absorption of small peptides because of
345 their proteolytic activity. However, recent works suggested that proline-rich peptides may resist the
346 action of brush-border peptidases and cross, intact, the intestinal barrier in Caco-2 cell system. For
347 example, the proline-rich β-casein-derived peptides NIPPLTQTPV exhibited a relative stability
348 towards brush-border membrane peptidases and is translocated intact into Caco-2 cells (Picariello et
349 al., 2013). In general, Picariello et al. (2013) found that the majority of the peptides resistant to
350 intestinal hydrolysis and able to cross the Caco-2 cells are proline-rich peptides.
351 Two peptides found in fraction F4, LxAP and PLx, are proline-containing peptides and therefore
352 potentially resistant to further digestion by brush-border exopeptidase. Indeed, it is now accepted

353 that significant amounts of small peptides can escape total digestion to amino acids and enter the
354 circulation intact (Vermeirssen et al., 2004).
355
356 **Conclusions**
357 In this study, for the first time, we demonstrated that the gastro-intestinal digestion of thermally-
358 treated *Phaseolus vulgaris* whole bean (pinto bean) released ACE-inhibitory peptides. We
359 identified, after ultrafiltration and RP-HPLC purification followed by nanoflowLC-ESI-QTOF-
360 MS/MS, eleven peptides with previously established ACE-inhibitory activity. These peptides are di-
361 or tri-peptides having the potential to survive the action of brush-border peptidases and lower the
362 blood pressure of hypertensive patients.

Declaration of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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Figure captions

Fig. 1. Degree of hydrolysis (DH%) of bean proteins after the different steps of *in vitro* digestion. Values represent means ± SD of triplicate digestions. Different letters indicate that the values are significantly different ($P < 0.05$).

Fig. 2. SDS-PAGE of bean proteins. Molecular weight standard is shown in lane 1. Protein pattern of pinto bean is shown in lane 2. Sample after salivary digestion of beans is shown in lane 3. Samples after gastric digestion are in lanes 4 (bean digested proteins) and 5 (control digestion with digestive enzymes but without beans). Samples after pancreatic digestion are in lanes 6 (bean digested protein) and 7 (control digestion with digestive enzymes but without beans).

Fig 3. UV-chromatograms of the low molecular weight peptidic fraction (<3 KDa) obtained from pinto beans subjected to consecutive gastro-pancreatic digestion. F1-F5 represent the collected fraction used for the identification of the ACE-inhibitory peptides.

Figure 1

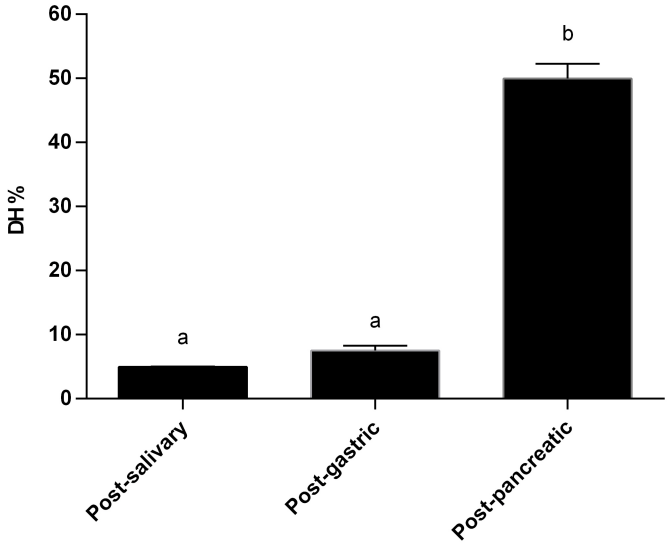
60
50
40
30
20
10
0
DH %
a
a
b
Post-salivary
Post-gastric
Post-pancreatic

Figure 2

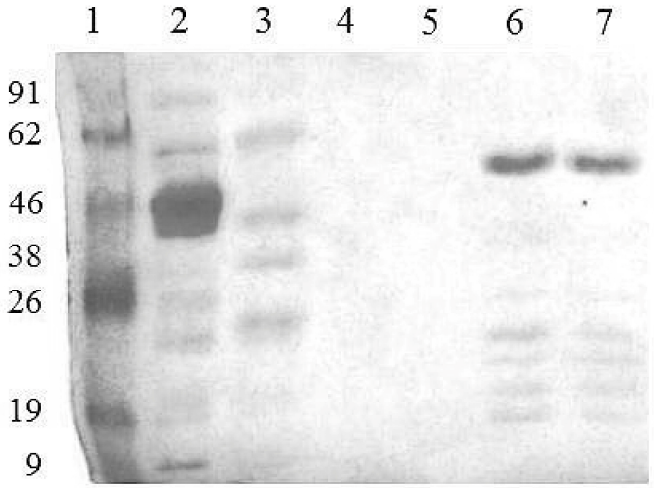

Figure 3

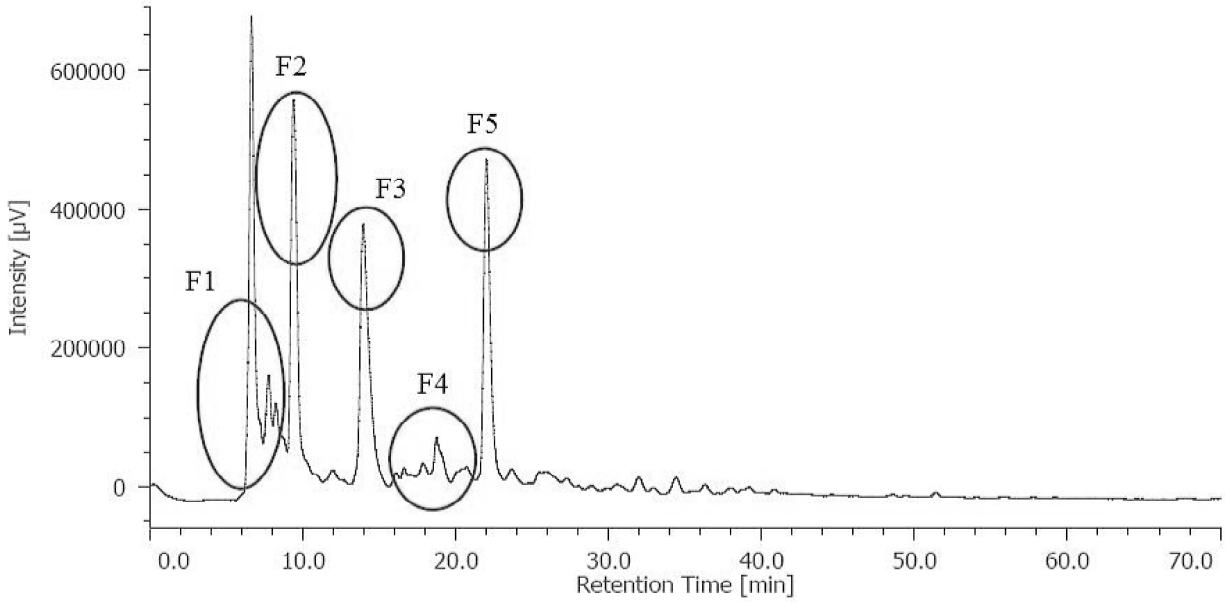
F1
F2
F3
F4
F5
600000
400000
200000
0
Intensity [µV]
0.0
10.0
20.0
30.0
40.0
50.0
60.0
70.0
Retention Time [min]

Table 1. Angiotensin-I converting enzyme (ACE)-inhibitory activity (IC_{50} values), peptides concentration and yield of the post-pancreatic < 3 kDa permeate and peptide fractions obtained through RP-HPLC purification of the post-pancreatic fraction.

	Peptides concentration (mg/mL)	*Estimated yield*[a] *(%)*	*IC_{50}*[b] *(µg peptides/mL)*
< 3 kDa permeate	4.53 ± 0.12	100	105.6 ± 2.1
HPLC F1	0.98 ± 0.05	21.7	6.4 ± 0.1
HPLC F2	0.67 ± 0.01	14.8	18.1 ± 1.0
HPLC F3	0.62 ± 0.02	13.7	5.5 ± 0.1
HPLC F4	0.56 ± 0.01	12.4	5.4 ± 0.2
HPLC F5	0.59 ± 0.02	13.0	15.1 ± 0.7

[a] yield was calculated as follow: (peptides concentration in the fraction)*100/ (peptides concentration in the post-pancreatic < 3 kDa permeate).
[b] IC_{50} is defined as the concentration of peptides needed to inhibit of 50% ACE activity.

Table 2. Peptides identified in the RP-HPLC fraction F1 of < 3 KDa permeate obtained from *Phaseolus vulgaris* after simulated gastro-intestinal digestion

Observed mass (m/z)	*Calculated mass*[a]	*ppm*	*Peptide sequence*[b]	*Protein precursor*	*Bioactivity*[c]	*Ref.*
622.220	622.220	0	EEEES	α and β subunits of phaseolin		
334.163	334.161	6.0	VSE	various proteins		
331.236	331.234	6.0	ALxK	α and β subunits of phaseolin		
279.159	279.170	39.4	LxF	α and β subunits of phaseolin and other proteins	ACE-inhibitor (LF IC_{50}= 349 µmol/L; IF IC_{50}= 930 µmol/L)	Meisel 1998; Cheung et al. 1980
269.160	269.161	-3.7	LxH	α and β subunits of phaseolin and other proteins	Antioxidant activity (LH)	Chen et al. 1996
247.131	247.139	-32.4	EV	α and β subunits of phaseolin and other proteins	ACE-inhibitor (IC_{50}=nd)	van Platerink et al. 2008
233.115	233.113	8.6	DV	α and β subunits of phaseolin and other proteins		
219.134	219.134	0	TV	various proteins		
205.120	205.118	9.8	SV	various proteins		
189.124	189.123	5.3	AV	various proteins		

[a]Monoisotopic mass
[b]Lx indicates leucine or isoleucine
[c]Potential bioactivities were achieved from the BIOPEP database

Table 3. Peptides identified in the RP-HPLC fraction F3 of < 3 KDa permeate obtained from *Phaseolus vulgaris* after simulated gastro-intestinal digestion

Observed mass (m/z)	*Calculated mass*[a]	*ppm*	*Peptide sequence*[b]	*Protein precursor*	*Bioactivity*[c]	*Ref.*
519.240	519.241	-1.9	NGVET	various proteins		
503.284	503.282	4.0	ALxDGK	α and β subunits of phaseolin and other proteins		
471.297	471.293	8.5	NLxPK	various proteins		
442.277	442.277	0	VAPR	various proteins		
375.188	375.187	2.7	ALxDG	α and β subunits of phaseolin and other proteins		
352.201	352.198	8.5	VPH	α and β subunits of phaseolin and other proteins		
346.195	346.197	-5.8	VVE	various proteins		
311.126	311.124	6.4	EY	α and β subunits of phaseolin and other proteins	ACE-inhibitor (IC_{50}= 2.7 µmol/L)	Wu et al. 2008
290.172	290.171	3.4	AVT	various proteins		
247.130	247.129	4.1	DLx	α and β subunits of phaseolin and other proteins		
233.150	233.150	0	TLx	α and β subunits of phaseolin and other proteins		
203.140	203.139	4.9	ALx	α and β subunits of phaseolin and other proteins	ACE-inhibitor (AI IC_{50}= 3.4 µmol/L); dipeptidyl peptidase IV inhibitor (AL IC_{50}= 882 µmol/L)	Nakahara et al. 2010; Nongonierma et al. 2013
189.124	189.123	5.3	GLx	α and β subunits of phaseolin and other proteins	ACE-inhibitor (GL IC_{50}= 2500 µmol/L; GI IC_{50}= 1200 µmol/L)	Cheung et al. 1980

[a]Monoisotopic mass
[b]Lx indicates leucine or isoleucine
[c]Potential bioactivities were achieved from the BIOPEP database

Table 4. Peptides identified in the RP-HPLC fraction F4 of < 3 KDa permeate obtained from *Phaseolus vulgaris* after simulated gastro-intestinal digestion

Observed mass (m/z)	*Calculated mass*[a]	*ppm*	*Peptide sequence*[b]	*Protein precursor*	*Bioactivity*[c]	*Ref.*
669.331	669.357	-38.8	VNPDPK	various proteins		
650.265	650.263	3.1	SGSGDEV	α and β subunits of phaseolin		
624.274	624.299	-40.0	FNEKS	various proteins		
600.343	600.335	13.3	AVEGPK	various proteins		
577.248	577.246	3.5	NLxDSE	various proteins		
437.268	437.251	38.9	LxAPH	various proteins		
409.173	409.172	2.4	FNE	various proteins		
400.257	400.255	5.0	VGPK	various proteins		
390.193	390.187	15.4	LxEE	various proteins		
389.202	389.203	-2.6	LxQE	various proteins		
376.174	376.171	8.0	EDLx	various proteins		
374.210	374.203	18.7	AVGGA	various proteins		
362.191	362.192	-2.8	LxET	various proteins		
360.216	360.213	8.3	LxVE	various proteins	ACE-inhibitor (LVE IC_{50}= 14.2 µmol/L)	Qian et al. 2007
359.229	359.229	0	VLxQ	various proteins		
300.192	300.192	0	LxAP	various proteins	ACE-inhibitor (LAP IC_{50}= 3.5 µmol/L; IAP IC_{50}= 2.7 µmol/L)	Fujita et al. 2000 ; Motoi and Kodama 2003
295.129	295.129	0	EF	various proteins	Renin inhibitor (IC_{50}= 22.7 µmol/L)	Li and Aluko 2010
281.113	281.113	0	DF	various proteins		
261.145	261.145	0	ELx	α and β subunits of phaseolin and other proteins		
253.118	253.118	0	SF	various proteins	ACE-inhibitor (IC_{50}= 130 µmol/L)	Meisel et al 2006
237.124	237.123	4.2	AF	α and β subunits of phaseolin and other proteins	ACE-inhibitor (IC_{50}= 190 µmol/L)	Cheung et al. 1980
231.170	231.170	0	VLx	various proteins		

229.155	229.155	0	PLx	α and β subunits of phaseolin and other proteins	ACE-inhibitor (IC_{50}= 337 µmol/L)	Byun and Kim 2002
223.108	223.108	0	GF	α and β subunits of phaseolin and other proteins	ACE-inhibitor (IC_{50}= 630 µmol/L)	Cheung et al. 1980

[a]Monoisotopic mass
[b]Lx indicates leucine or isoleucine
[c]Potential bioactivities were achieved from the BIOPEP database