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Title: Identification, functional gastrointestinal stability and molecular docking studies of lentil peptides with dual antioxidant and angiotensin I converting enzyme inhibitory activities

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Keywords: ACE inhibitory activity, antioxidant activity, lentil peptides, gastrointestinal digestion, molecular docking

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Abstract: The objective was to identify peptides with dual antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities released from lentil proteins by Savinase®. The influence of gastrointestinal digestion on peptide bioactivity was also assayed. Fragments from vicilin, convicilin and legumin were the most abundant peptides identified. Peptides LLSGTQNQPSFLSGF, NSLTLPILRYL, TLEPNSVFLPVLLH showed the highest antioxidant (0.013-1.432 μmol Trolox eq./ μmol peptide) and ACE inhibitory activities (IC_{50} =44-120 μM). Gastrointestinal digestion of peptides improved their dual activity (10-14 μmol Trolox eq./ μmol peptide; IC_{50} =11-21 μM). In general, C-terminal heptapeptide was crucial for their dual activity. ACE inhibition relies on the formation of hydrogen bonds between C-terminal residues of lentil peptides and residues of the ACE catalytic site. The present study helps clarifying the relationship between structure and dual antioxidant/antihypertensive activity of lentil peptides opening new opportunities to food industry such as the application of lentil protein hydrolysates as ingredients for development of functional foods.

HIGHLIGHTS

- Lentil peptides showing potential antihypertensive activity were identified
- Bioactive fragments derived from lentil vicilin, convicilin and legumin
- Gastrointestinal digestion of peptides improved markedly their bioactivity
- C-terminal heptapeptide was crucial for antioxidant and ACE inhibitory activities
- ACE inhibition relies on hydrogen bonds involving residues of the catalytic site

1 ABSTRACT

2 The objective was to identify peptides with dual antioxidant and angiotensin I converting enzyme
3 (ACE) inhibitory activities released from lentil proteins by Savinase®. The influence of
4 gastrointestinal digestion on peptide bioactivity was also assayed. Fragments from vicilin, convicilin
5 and legumin were the most abundant peptides identified. **Peptides LLSGTQNQPSFLSGF,**
6 **NSLTLPIRLYL, TLEPNSVFLPVLLH showed the highest antioxidant (0.013-1.432 μ mol Trolox**
7 **eq./ μ mol peptide) and ACE inhibitory activities (IC_{50} =44-120 μ M). Gastrointestinal digestion of**
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9 **general, C-terminal heptapeptide was crucial for their dual activity. ACE inhibition relies on the**
10 **formation of hydrogen bonds between C-terminal residues of lentil peptides and residues of the ACE**
11 **catalytic site. The present study helps clarifying the relationship between structure and dual**
12 **antioxidant/antihypertensive activity of lentil peptides opening new opportunities to food industry**
13 **such as the application of lentil protein hydrolysates as ingredients for development of functional**
14 **foods.**

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16 molecular docking

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19 1. Introduction

20 Hypertension is a chronic disease characterized by insufficient relaxation of blood vessels which is
21 associated to an increased risk of cerebrovascular and cardiovascular diseases. These diseases affect
22 millions of people annually, resulting in extensive morbidity and mortality. Human blood pressure is
23 regulated by systemic renin-angiotensin system (RAS) that relies on a proteolytic cascade involving
24 two key proteases, namely, renin (EC 3.4.23.15), an aspartyl protease secreted into the blood from
25 the liver, and angiotensin I converting enzyme (ACE; EC 3.4.15.1), a carboxypeptidase present on
26 the external surface of endothelial cells of the lung (Natesh, Schwager, Sturrock, & Acharya, 2003).
27 Renin **converts angiotensinogen, a serum α 2-globulin** produced in the liver, into the inactive
28 decapeptide angiotensin I. This peptide is hydrolyzed by ACE to form the vasoactive octapeptide
29 angiotensin II to cause vasoconstriction (Pacurari, Kafoury, Tchounwou, & Ndebele, 2014). ACE
30 also inactivates bradykinin, a vasodilatory peptide that contributes to blood pressure elevation.
31 Hypertension has been related to increased activity of renin and/or ACE and high blood angiotensin
32 II levels (Aluko, 2015a). Besides RAS, other important mechanism to regulate blood pressure is the
33 production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) (Török, 2008). NO is a
34 vasodilatory molecule that helps to maintain normal blood pressure levels. Studies have shown that
35 inadequate antioxidant protection or excessive production of reactive oxygen species (ROS) create a
36 condition known as oxidative **stress that lead, for instance, to changes in eNOS activity, reduction of**
37 **NO bioavailability and impaired vasodilatory** responses (Harrison & Gongora, 2009). Traditional
38 drug therapies for hypertension have involved the use of ACE or renin inhibitors (Abassi, Winaver,
39 & Feuerstein, 2009). More recently, antioxidant agents have also been proven to **increase NO**
40 **bioavailability and to prevent the damage that ROS cause in patients with hypertension (Semen et al.,**
41 **2016).** On the basis of the multiple hypertension-inducing factors, future prospects are focused on
42 developing new **agents targeting multiple pathways including vasoconstriction, oxidative stress and**
43 **inflammation.**

44 Because antihypertensive drugs can cause negative side effects, they are believed to reduce patients
45 compliance, leading eventually to exacerbated disease conditions, and increased health care costs.
46 Therefore, there has been increased interest in developing natural compounds that could replace or
47 complement antihypertensive drugs due to reduced side effects associated to their consumption
48 (Aluko, 2015b). Natural peptides exhibiting significant potency to reduce blood pressure in
49 preclinical and clinical studies have been produced from several food-derived proteins through
50 enzymatic hydrolysis, fermentation or gastrointestinal digestion (Aluko, 2015a). In particular,
51 legume storage proteins (mainly 11S and 7S globulins) are abundant sources of bioactive peptides
52 (Roy, Boye, & Simpson, 2010) that have been used as raw materials for the production of
53 hydrolysates with potential antihypertensive effects (Betancur-Ancona, Sosa-Espinoza, Ruiz-Ruiz,
54 Segura-Campos, & Chel-Guerrero, 2014; Aluko et al., 2015); however, the identification of peptides
55 responsible for the biological function is still limited. Recent approaches are being made in the field
56 of production, such as the selection of proteolytic enzymes that facilitate the production of active
57 fragments from legume proteins (Rui, Boye, Simpson, & Prasher, 2012). In this sense, our previous
58 studies have shown that among five food-grade enzymatic preparations, Savinase® 16 L was the
59 most effective to produce multiactive hydrolysates with antioxidant, ACE inhibitory and anti-
60 inflammatory activities from lentil and bean protein concentrates (Garcia-Mora et al., 2015; Garcia-
61 Mora, Peñas, Frias, & Martínez-Villaluenga, 2014). Despite the potential bioactive properties, there
62 is still a scarcity of **information regarding structure-function relationships for legume-derived**
63 **peptides. Latest approaches using chemometric and chemoinformatic tools could be very helpful in**
64 **the analysis of food-derived bioactive peptides and also to design peptides with desired bioactivities**
65 (Iwaniak, Minkiewicz, Darewicz, Protasiewicz, & Mogut, 2015).

66 The main objective of this study was to identify lentil-derived peptides with dual antioxidant and
67 ACE inhibitory activity in order to propose them as ingredients for functional food design. To exert a
68 positive health effect, bioactive peptides functionality must be maintained upon gastrointestinal

69 digestion. Therefore, we have determined functional stability of peptides after simulated
70 gastrointestinal digestion. C-terminal heptapeptides residues were synthesized to determine
71 important regions in the peptide sequence and their physicochemical properties for dual activity. We
72 have also used molecular modelling to predict human somatic ACE in complex with the most potent
73 lentil-derived ACE inhibitory peptides.

74 2. Materials and Methods

75 2.1. Materials

76 Lentil seeds (*Lens culinaris* var. Castellana) were provided by Semillas Iglesias S.A. (Salamanca,
77 Spain) and stored in polyethylene bins at 4 °C. Commercial food grade Savinase® 16 L, an alkaline
78 serine protease from *Bacillus* sp., was kindly provided by Novozymes (Bagsvaerd, Denmark).
79 Tripeptide Abz-Gly-Phe(NO₂)-Pro was purchased from Cymit-Quimica (Barcelona, Spain). O-(6-
80 Chlorobenzotriazol-1-yl)-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HCTU),
81 N,N-diisopropylethylamine (DIEA), trifluoroacetic acid (TFA), triisopropylsilane (TIPS), Fmoc-His
82 (Trt)-wang resin (0.8 mmol/g) and Fmoc-Asn (Trt)-wang resin (0.8 mmol/g) were from Sigma-
83 Aldrich (Madrid, Spain). N-Fluorenyl-9-methoxycarbonyl (Fmoc) protected L-amino acids (Fmoc-
84 Ala-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Asp(OtBu)-OH, Fmoc-Asn(Trt)-OH, Fmoc-Gln(Trt)-OH,
85 Fmoc-Glu(OtBu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-
86 Lys(Boc)-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(Trt)-OH, Fmoc-Thr(Trt)-OH, Fmoc-
87 Tyr(tBu)-OH, Fmoc-Val-OH), Fmoc-Phe-wang resin (0.86 mmol/g), Fmoc-Leu-wang resin (0.64
88 mmol/g) were provided by Novabiochem (Madrid, Spain). N,N-Dimethylformamide (DMF) and
89 dichloromethane (DCM) were dried and purified by standard methods. All other chemicals were
90 purchased from Sigma-Aldrich (Madrid, Spain) unless otherwise specified.

91

92 2.2. Preparation of lentil hydrolysates

93 Lentil proteins were isolated using alkaline extraction as described previously (Garcia-Mora et al.,
94 2014). Freeze-dried lentil proteins were suspended in deionized water (2%, w/v), equilibrated at 40
95 °C and adjusted to pH 8 with 1 M NaOH. Savinase® 16 L was added at a final enzyme to substrate
96 ratio (E/S) of 0.1 U/mg of soluble lentil protein. Hydrolysis was performed at 40 °C for 2 h with the
97 pH maintained in the range 7.9–8.3 by the addition of 1 M NaOH. Reactions were finalized by
98 heating samples at 80 °C for 15 min. Hydrolysates were ultrafiltrated using Amicon ultracentrifugal
99 filter units of 3000 Da molecular weight cut off (Sigma-Aldrich, Madrid, Spain). Filtrates were
100 freeze dried for further analysis (67% weight yield).

101 2.3. Analysis of lentil hydrolysates by on-line nanoUPLC–ESI-MS/MS

102 For peptide identification, 10 µg of lentil 3 kDa filtrates were desalted using a reverse phase
103 (PorosR2©) column with 80% acetonitrile/0.1% trifluoroacetic acid. Later, the peptides were dried
104 and resuspended with 10 µL 0.1% TFA. An aliquot of 5 µL of resuspended sample was injected in
105 the linear trap quadrupole Orbitrap Velos (Thermo Fisher Scientific, Madrid, Spain). Peptides were
106 loaded onto a C18-A1 ASY-Column 2 cm precolumn (Thermo Fisher Scientific, Madrid, Spain) and
107 then eluted onto a Biosphere C18 column (inner diameter 75 µm, 15 cm long, 3 µm particle size)
108 (NanoSeparations) at a flow rate of 250 nL/min on a nanoEasy high-performance liquid
109 chromatography (Proxeon, Madrid, Spain) coupled to a nanoelectrospray ion source (Proxeon,
110 Madrid, Spain). The mobile phases used consisted of 0.1% formic acid in 2% acetonitrile (solvent A)
111 and 0.1% formic acid in 100% acetonitrile (solvent B). The solvent gradient was from 0% B to 35%
112 B in 140 min. Mass spectra was acquired in the positive ion mode. Full-scan MS spectra (m/z 400–
113 1800) were acquired with a target value of 1,000,000 at a resolution of 30,000 at m/z 400 and the 15
114 most intense ions were selected for collision induced dissociation fragmentation in the linear trap
115 quadrupole with a target value of 10,000 and normalized collision energy of 38%. Precursor ion
116 charge state screening and monoisotopic precursor selection were enabled. Singly charged ions and
117 unassigned charge states were rejected. Dynamic exclusion was enabled with a repeat count of 1 and

118 exclusion duration of 30 s. Proteome Discoverer 1.4.288 (Thermo Fisher Scientific, Madrid, Spain)
119 with MASCOT 2.3 was used to search the NCBI database, taxonomy Viridiplantae (1530236
120 sequences). Database search parameters used were the following: peptide tolerance, 10 ppm;
121 fragment ion tolerance, 0.8 Da; none enzyme and variable modification, methionine oxidation. In all
122 peptide identifications, the probability scores were greater than the score fixed by Mascot as
123 significant with a p -value < 0.05 . The automatic decoy database search function of Protein Discover
124 was enabled to allow estimation of false discovery rate (FDR).

125 2.4. Solid-phase peptide synthesis

126 2.4.1. *Coupling procedure.* The corresponding Wang resin (100 mg), previously swollen in DMF and
127 DCM (1 mL/100 mg of resin, four times for 30 s), was treated with 20% piperidine in DMF (once for
128 5 min) and (once for 20 min), for Fmoc removal, and washed with DMF/DCM/DMF/DCM/DMF (4
129 times for 0.5 min). The appropriate Fmoc amino acid (2 equiv.) was added to the resin along with
130 HCTU/DIEA (2 equiv.) in dry DMF (1 mL/100 mg of resin) at room temperature for 1 h and then
131 washed with DMF/DCM/DMF/DCM/DMF (4 times for 0.5 min). The cycle described above is then
132 repeated for each amino acid until the target peptide was completely synthesized. Each coupling was
133 monitored by the ninhydrin or chloranile tests and, when necessary, repeated with a fresh portion of
134 Fmoc-amino acid and the indicated coupling reagents. The N-terminal Fmoc residue was finally
135 removed with 20% piperidine in DMF (once for 5 min) and (once for 20 min), and the resin was
136 washed with DMF/DCM/DMF/DCM (4 times for 0.5 min) and dried (water vacuum pump suction at
137 room temperature for 30 min).

138 2.4.2. *Cleavage step.* Cleavage of peptides from the resin, and concomitant deprotection of side-
139 chains, was performed using TFA/H₂O/TIPS (95:2.5:2.5) (1 mL/100 mg of resin) at room
140 temperature for 3 h. The resin was filtered off and crude products were precipitated with cold diethyl
141 ether. The resulting solid was centrifuged and washed twice with ether (5,000 rpm at 15°C for 10
142 min), and then dissolved in H₂O or mixtures of H₂O/acetonitrile and lyophilized.

143 *2.4.3. Peptide purification and characterization.* Peptides were purified by MPLC using SNAP
144 cartridges (12 g KP-C18-HS) in an ISOLERA ONE (Biotage, Madrid, Spain). A gradient of
145 CH₃CN:H₂O (0.05% TFA) from 0:100 to 30:70 over 60 min, as mobile phase, and a flow of 5
146 mL/min were used. Some peptides were purified by semipreparative RP-HPLC-MS 2545 (Waters,
147 Madrid, Spain) coupled to a mass spectrometer 3100 detector, using a SUNFIRETM column C18 (5
148 µm, 10 x 150 mm) and an 8 mL/min flow of a suitable CH₃CN (0.1% HCO₂H) [Solvent A]:H₂O
149 (0.1% HCO₂H) [Solvent B] gradient as mobile phase. The purity of peptides was analyzed using an
150 analytical HPLC model 1120 Compact LC (Agilent, Madrid, Spain) equipped with an Eclipse Plus
151 column C18 (4.6 x 150 mm), at 1.5 mL/min flow, and a gradient from 5% to 50% CH₃CN [Solvent
152 A]:H₂O (0.05% TFA) [Solvent B] gradient as mobile phase, over 20 min. Characterization of the
153 products was performed by ESI-MS Micromass ZQ 2000 (Waters, Madrid, Spain). Details are
154 recorded in Supplementary Table 1.

155 *2.5. Oxygen radical absorbance capacity*

156 Oxygen radical absorbance capacity (ORAC) values of each peptide were determined in triplicate
157 following a previously reported method (Garcia-Mora et al., 2014). Briefly, reactions were
158 performed using 20 µL Trolox standard (1–160 µM), or peptides or 20 µL blank (75 mM phosphate
159 buffer pH 7.4), 120 µL of 116.9 nM fluorescein (final concentration 70 nM/well) and 60 µL of 40
160 mM AAPH per well. A black 96-well plate was read at $\lambda_{\text{emission}}$ of 485 nm and $\lambda_{\text{excitation}}$ of 582 nm
161 every 2 min at sensitivity 60 and 37 °C using Synergy HT plate reader (Biotek, Winooski, VT).
162 Results were expressed as Trolox equivalents (TE, in µmol Trolox eq./µmol peptide) and calculated
163 using the generated Trolox standard curve.

164 *2.6. Determination of ACE inhibitory activity*

165 Peptide ACE inhibition assay was determined in triplicate using a previously reported method
166 (Garcia-Mora et al., 2014). The generated fluorescence was read every minute for 30 min at emission

and excitation wavelengths of 355 and 405 nm, respectively, in a fluorometer (Biotek, USA). ACE inhibitory activity was expressed as the peptide concentration needed to inhibit 50% of ACE activity (IC_{50}). IC_{50} values were determined by dose-response curves in which the range of concentrations (0–500 μ M) was distributed in a logarithmic scale and using the nonlinear regression sigmoidal curve fit function in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).

2.7. Simulated gastrointestinal digestion

Synthesized peptides were subjected to a sequential gastric and duodenal digestion according to the method described by Garcia-Mora et al. (2014). Briefly, peptides (10 mg) were dissolved in 3 mL of simulated gastric fluid (SGF, 0.15 M NaCl; pH 2.5). Samples were pre-heated for 15 min at 37 °C. Then 400 μ L of a solution containing 0.59% (w/v) porcine pepsin (EC 3.4.23.1, 3640 U/mg protein) in SGF were added and the pH was adjusted to 2.5 with 0.01 M HCl. Digestion was performed at 37 °C for 2 h and subsequently stopped by increasing the pH to 7.5 with 0.1 M NaOH. Gastric digests were adjusted to pH 6.5 with 0.01 M HCl. In order to simulate a duodenal environment the following solutions were added: 150.8 μ L of a bile salt mixture containing equimolar quantities (0.125 M) of sodium taurocholate and glycodeoxycholic acid (Sigma-Aldrich, Madrid, Spain), 46.08 μ L of 1 M $CaCl_2$ (Merck, Darmstadt, Germany), 500 μ L of 0.25 M Bis-Tris (pH 6.5), and 100 μ L of pancreatin from porcine pancreas (EC number 232.468.9) in SFG at pH 7.0. Digestion was carried out at 37 °C for 2 h. Finally, pancreatin was inactivated by heating at 80 °C for 15 min.

2.8. Molecular Docking, IFD Schrödinger.

Ligands were built using Maestro and prepared using Ligprep module from Maestro 9.2 (Schrödinger Inc., München, Germany), with the OPLS2005 force field (Sherman, Beard, & Farid, 2006; Sherman, Day, Jacobson, Friesner, & Farid, 2006). Two crystal structures were used in these studies of pdb codes 4APH and 4APJ, corresponding to somatic ACE (sACE) bound to Angiotensin II (AngII) and bradikinin (BPPb), respectively (Masuyer, Schwager, Sturrock, Isaac, & Acharya,

2012). The protein was prepared using the protein preparation wizard within Maestro. Molecular docking studies were performed using the induced fit docking protocol within the Schrödinger suite, this protocol is based in Glide 5.7 and Prime 3.0. In the first step, ligands were docked to the rigid protein using the soften-potential docking with a van der Waals radii scaling of 0.5. Glide grid was positioned on the centroid of AngII or BPPb. The resulting top 20 poses of ligands were used to perform a conformational search and minimization of the sidechain of ACE residues within 5 Å of the ligand with the Prime module. The ACE-peptide complexes whose energy is within 30 kcal/mol of the global minimum were subject to a re-docking with the Glide module. In this way, the flexibility of the protein around the ligand binding site is considered.

2.9. *Molecular Docking, simulated annealing molecular dynamics*

The analysis of the IFD studies allowed the selection of three docking binding poses for peptides **1** and **3**, and two for peptide **4**. The selected complexes were subjected to molecular dynamics simulations, which allows considering the flexibility of the whole complexes. Each ACE-peptide complex was solvated with TIP3P water molecules and neutralized by Na⁺ counterions. Molecular dynamics simulations were performed with periodic boundary conditions using Amber and the FF12SB forcefield (Pearlman et al., 1995; Case et al., 2012). Initially a 5000 step steepest descent, followed by 5000-steps conjugate gradient minimization was performed to minimize steric clashes. Then, the system was heated from 0 K to at 300 K gradually over 100 ps, using the positional restraint method, starting with a restraint weight of 5.0 Kcalmol⁻¹Å⁻² and an integration time step of 1.0 fs. First, the solutes were restrained while the water and counter-ions were equilibrated in the first 25 ps, then, progressively weaker restraints were applied till all were removed in the last 100 ps under the NPT ensemble. The temperature was maintained at 300 K using Langevin dynamics. For the production run an integration time step of 2.0 fs was used, while hydrogen bonds were constrained using the SHAKE algorithm. The particle Mesh Ewald method was used for the calculation of electrostatic interactions. A 5 ns production run was then carried out, and the

216 evaluation of the ACE-heptapeptide complex allowed the selection of one conformation for each
217 peptide, and for the selected complexes the production run was carried out till 40 ns. The PyMOL
218 Molecular Graphic System v1.7.0.1 (Schrödinger Inc., München, Germany) was used for
219 visualization and making figures.

220 2.10. Data analysis

221 Statistical analysis was conducted using Statgraphics Centurion XVI software, version 16.1.17
222 (Statistical Graphics Corporation, Rockville, MD, USA). Group mean comparisons were conducted
223 using Duncan's multiple-range test and were considered significant at $P < 0.05$ based on the
224 minimum significant differences from one-way analysis of variance (ANOVA) with $\alpha = 0.05$.
225 Results are presented as means $\pm$ standard deviation. All analyses were done at least in triplicate.

226

227 3. Results and Discussion

228 3.1. Identification of most abundant peptides from 3 kDa permeates of lentil hydrolysate

229 Previous work in our laboratory demonstrated that 3 kDa permeates from lentil hydrolysates
230 produced by Savinase® 16 L for 120 min exert an inhibitory effect ($IC_{50} = 0.18$ mg/mL) on ACE
231 activity (Garcia-Mora et al., 2014). Most abundant peptides in this fraction were identified by liquid
232 chromatography coupled to tandem mass spectrometry (Table 1). A total of 8 peptides were found,
233 corresponding to the major seed storage proteins: 7S and 11S globulins. BLAST results indicated
234 that peptides DLAIPVNRPGQLQSF (peptide 1), LLSGTQNQPSFLSGF (peptide 2),
235 DLAIPVNNPGQLESF (peptide 5), RFQTIYNENGHIRLL (peptide 6) and
236 EITPEKNPQLQDLIDIFVN (peptide 8) matched sequences present in allergens Len c1.0101 and/or
237 c1.0102, whereas NSLTLPILRYL (peptide 3), TLEPNSVFLPVLLH (peptide 4),
238 LVNEGKGNLELVGF (peptide 7) matched sequences of legumin type B, vicilin-like antimicrobial
239 peptides 2-3 and convicilin, respectively. In addition, peptides 1 and 8 were also fragments

240 corresponding to vicilin. Most of the identified peptides contained C-terminal residues of Phe (F) and
241 Leu (L), whereas only 2 peptides showed His (H) and Gln (N) as C-terminal residues. This
242 observation could be explained on the basis of the broad specificity of *Bacillus* sp. alkaline serine
243 proteases and their preferences for aromatic or hydrophobic residues at position P1 of the cleavage
244 site. All identified peptides were custom synthesized by solid-phase in order to confirm or disprove
245 their dual antioxidant and ACE inhibitory activity.

246 3.2. *Oxygen radical scavenging activity of lentil-derived peptides*

247 Antioxidant activity of synthesized peptides was determined by the ORAC assay based on a
248 hydrogen atom transfer mechanism. This method uses a radical initiator (ABTS) to generate peroxy
249 radical (ROO•) that abstracts a hydrogen atom from antioxidant molecules present in samples.
250 ORAC values of synthesized peptides varied from 0.004 to 1.43 $\mu\text{mol Trolox eq./}\mu\text{mol peptide}$
251 (Table 2). The antioxidant activity of synthetic lentil-derived peptides was within the range of
252 reported antioxidant activity for amaranth 11S globulin (0.06-6.07 $\mu\text{mol Trolox eq./}\mu\text{mol peptide}$)
253 (Orsini Delgado et al., 2016), casein (0.0025-3.22 $\mu\text{mol Trolox eq./}\mu\text{mol peptide}$) (Contreras,
254 Carrón, Montero, Ramos, & Recio, 2009; Contreras, Sanchez, Sevilla, Recio, & Amigo, 2013) and
255 ovotransferrin peptides (0.43-15.47 $\mu\text{mol Trolox eq./}\mu\text{mol peptide}$) (Shen, Chahal, Majumder, You,
256 & Wu, 2010) and lower than values reported for β -lactoglobulin peptides (4.45-7.67 $\mu\text{mol Trolox}$
257 $\text{eq./}\mu\text{mol peptide}$) rich in aromatic amino acids such as Tyr (Y) and Trp (W) considered as
258 determinants in the radical scavenging activity of food derived peptides (Hernández-Ledesma,
259 Amigo, Recio, & Bartolomé, 2007).

260 Using a dataset of reported ORAC values for peptides containing from 2 to 20 residues, quantitative
261 structure-activity relationship (QSAR) models have revealed that properties of C-terminal regions
262 are more important than those at N-terminal for antioxidant activity (Li & Li, 2013). Therefore, to
263 ascertain if C-terminal sequences of peptides identified in lentil were the determinants for their
264 antioxidant activity, C-terminal heptapeptide residues were synthesized and their antioxidant activity

265 tested (Table 2). Different results were observed with the deletion of the N-terminal region for each
266 peptide. Peptides Ct-1, Ct-4 and Ct-7 showed a significantly lower antioxidant activity than their
267 parent peptides 1, 4 and 7 ($P<0.05$) indicating that amino acids at N-terminal regions are also
268 contributing to the antioxidant activity of parent peptides. In contrast, Ct-2 showed similar ORAC
269 values than peptide 2, whereas Ct-3 and Ct-5 showed values more than 2-fold higher than their
270 parent peptides 3 and 5, respectively ($P<0.05$). These results indicate that for peptides 2, 3 and 5, the
271 N-terminal region is less important for antioxidant activity as it has been previously described using
272 QSAR models (Li & Li, 2013).

273 The analysis of amino acids by the ORAC method in earlier studies have indicated that several
274 amino acids, in the following order, Trp (W), Tyr (Y), Met (M), Cys (C), His (His) and Phe (F) have
275 antioxidant properties which increase when they are incorporated into peptides (Saito *et al.*, 2003;
276 Shen *et al.*, 2010). Plausible mechanisms of radical scavenging activity of amino acid residues in
277 peptides include proton or hydrogen atom donation. Neutralization of peroxy radicals in the ORAC
278 assay by Tyr (Y), Trp (W) and Cys (C) are attributed to the donation of phenolic, indolic and
279 sulfhydryl hydrogen, respectively (Hernández-Ledesma, Miralles, Amigo, Ramos, & Recio, 2005;
280 Elias, Kellerby, & Decker, 2008). His (H) has been also commonly associated with antioxidant
281 activity due to its hydrogen-donating and radical-trapping imidazole ring (Elias, Kellerby, & Decker,
282 2008). The presence of Tyr (Y) or His (H) in peptides 3, 4, and in their C-terminal heptapeptide
283 sequences, together with Ct-6 may account for their higher antioxidant activity.

284 Moreover, it has been found that key sequence positions are significant for antioxidant activity of
285 food-derived peptides. Studies focused on antioxidant peptides derived from egg and dairy proteins
286 support the importance of bulky hydrophobic amino acids (Trp, Tyr, Ile, Leu, Met, Phe) in the
287 sequence at C₃ when antioxidant activity is measured by ORAC assay (Hernández-Ledesma *et al.*,
288 2005; Hernández-Ledesma *et al.*, 2007; Shen *et al.*, 2010). Similarly, QSAR models have indicated
289 that the stericity of bulky amino acids at position C₄, N₂ and C₃ is related positively to antioxidant

activity (Li & Li, 2013). Considering the C-terminal sequence from heptapeptides, it can be observed that lentil derived peptides presented several bulky residues, not always in these positions, although in general there is one at C₄ and three of them have a bulky group at N₂.

3.3. *ACE inhibitory activity of lentil-derived peptides*

ACE inhibitory activity (IC₅₀ value) of synthesized lentil-derived peptides is reported in Table 2. Peptides 1 and 5-8 showed no inhibitory activity in the concentration range tested (IC₅₀ > 250-500 μM). Peptide 3 showed the most potent activity (77 μM) followed by peptides 2 and 4 with similar IC₅₀ values (118 μM on average). Potency of these peptides was higher than values reported for pea dipeptides (2.25-7.23 mM) and similar or within the range reported in the literature for common bean (17.5-191.5 μM) and hemp seed (27-574 μM) peptides (Aluko et al. 2015; Luna-Vital, Gonzalez de Mejia, Mendoza, & Loarca-Pina, 2015). The ability of these peptides to inhibit ACE is consistent with an earlier study showing that hydrophobic or aromatic amino acid residues or Pro (P) at the C-terminus positively contributed to the improvement of ACE inhibitory potency (Wu et al., 2006). This previous study also concluded that C-terminal tetrapeptide residues for long-chain peptides containing from 4 to 10 residues is more important for their potency than the C-terminal tripeptide, indicating the relevance of the fourth amino acid residue, however, exact length of the C-terminal region to efficiently inhibit ACE activity is still unknown. According to this information, C-terminal heptapeptides of lentil-derived peptides were synthesized and their ACE inhibitory activity was analyzed (Table 2). Deletion of the N-terminal region significantly increased the potency of all lentil-peptides, which confirmed findings from previous studies (Wu et al., 2006; Zhou et al., 2013; Aluko, 2015b). The most active C-terminal heptapeptides were Ct-3 (15 μM), Ct-4 (25.7 μM) and Ct-1 (51.7 μM).

Required amino acids in the C-terminal region of long peptides for strong ACE inhibitory activity have been reported using structure-activity relationship studies (Aluko et al., 2015); however, there are still major differences in key amino acid positions which may be due to variations in modeling

tools and peptide databases. There is some agreement with regard to the presence of a positive charge, bulky hydrophobic and aromatic amino acid residues in the C-terminal tetrapeptide, requirements that are completely fulfilled for peptides Ct-3 and Ct-4 and are consistent with their higher potencies. Furthermore, on the basis of other studies, the presence of a branched-chain aliphatic amino acid such as Leu (L) at N-terminus could have also been responsible for the higher potency of Ct-3 compared to Ct-4.

3.4. Effect of gastrointestinal digestion on antioxidant and ACE inhibitory activity of lentil peptides

Bioactive peptides must resist gastrointestinal enzymes degradation to reach their target organs and tissues and exert their beneficial effects *in vivo*. Human trials have provided evidence for bioavailability of high molecular weight food-derived peptides. For example, soybean peptide lunasin (5 kDa) was found in human plasma at a concentration range from 0.05 to 0.12 mg/mL after 30 min and 1 h of ingestion of soybean products (Dia, Torres, de Lumen, Erdman, & de Mejía, 2009). Results of this study demonstrate that large oligopeptides can be absorbed in the intestine and reach target organs. Paracellular route has been found as the main intestinal-transport pathway for high MW peptides (1600–500 Da) (Wang & Li, 2017). Additionally other studies have reported transcytosis as an epithelial transport mechanism for a 17-residues peptide of bovine β -casein f(193–209) (Regazzo et al. 2010).

Because potential antioxidant and antihypertensive activities of peptides is dependent on amino acid composition and sequence, the loss of amino acid residues as a result of proteolysis within the gastrointestinal tract can lead to reduced or increased potency, or to a complete loss of activity. To confirm the gastrointestinal stability, lentil-derived peptides were treated *in vitro* with pepsin followed by pancreatin at 37 °C under appropriate pH conditions. A comparison of the antioxidant and ACE inhibitory activities of peptides before and after simulated gastrointestinal digestion (SGD)

339 is shown in Table 3. In general, the action of digestive enzymes increased the antioxidant and ACE
340 inhibitory activity of lentil-derived peptides. These results agree with our previous studies on
341 gastrointestinal digestion of lentil hydrolysates obtained with Savinase (Garcia-Mora et al., 2014;
342 Garcia-Mora, Peñas, Frias, Gomez, & Martinez-Villaluenga, 2015). The release of smaller peptide
343 fragments and amino acids from lentil oligopeptides might result in additive and synergistic
344 biological effects.

345 It is noteworthy to mention the potent dual activities observed in digests obtained from peptides 1, 2,
346 3 and 4 (9.66-14.29 $\mu\text{mol Trolox eq./}\mu\text{mol peptide}$; IC_{50} =11.4-21.25 μM ; for antioxidant and ACE
347 inhibitory activities, respectively). Antioxidant activity of gastrointestinal digests was notably higher
348 than values reported for casein (1.38-4.16 $\mu\text{mol Trolox eq./}\mu\text{mol peptide}$) and amaranth (0.06-6
349 $\mu\text{mol Trolox eq./}\mu\text{mol peptide}$) peptides derived from gastrointestinal digestion (Contreras et al.,
350 2013; Orsini Delgado et al., 2016). Similarly, ACE inhibitory potency was found within the range of
351 reported values for potent plant protein-derived peptides (IC_{50} =10-50 μM) (Aluko, 2015b). The most
352 popular commercial functional foods claiming antihypertensive effects contain lactotripeptides VPP
353 and IPP. IC_{50} values of these lactotripeptides (5 and 9 μM for IPP and VPP, respectively) are
354 comparable to peptide digests from peptides 1 and 3.

355 Table 4 shows all the possible hydrolysis products resulting from complete peptide degradation of
356 lentil peptides 1-8 by the gastrointestinal proteases and peptidases available in the PeptideCutter tool
357 of ExPASy. Some of the peptide sequences released from peptides 2, 3, 4, 6 and 8 such as QS, PIL,
358 TL, PVL, IR and DI corresponded to C-terminal or entire sequences of previously reported ACE
359 inhibitory and antioxidant peptides documented in the BIOPEP database which is consistent with the
360 increased bioactivity of the gastrointestinal digests. In addition, 5 known dipeptidyl peptidase
361 inhibitory peptides released from peptides 1, 3, 6, 7 and 8 were also predicted to be released during
362 gastrointestinal digestion of lentil Savinase hydrolysates. This finding encourages more research

363 studies to demonstrate their beneficial effects on insulin secretion and satiety regulation (Reinehr,
364 Roth, Enriòri, & Masur, 2010).

365 A limitation of this study is that peptide fragments and amino acids presented in Table 4 are based on
366 in silico gastrointestinal digestion. It is possible that including lentil peptides in a food matrix their
367 hydrolysis rate might be affected and therefore food digests reaching the intestine may contain intact
368 lentil peptides and a mixture of peptide fragments and amino acids derived from digestion in the
369 upper parts of the gut. *In vivo* studies on lentil peptides bioavailability and biological effects would
370 add further support on application of lentil hydrolysates as bioactive ingredients for the development
371 of functional foods.

372 3.5. *Molecular docking studies*

373 In order to get insights into the binding mode of lentil peptides into ACE, molecular docking studies
374 have been carried out for the most active C-terminal heptapeptides Ct-1, Ct-3 and Ct-4, as our results
375 indicated that this region is the most important for ACE inhibition. We performed docking studies
376 using two different reported crystal structures of the C-domain sACE, one in complex with the
377 vasoactive octapeptide angiotensin II (AngII, DRVYIHPF) and the other in complex with an eleven
378 amino acid inhibitor, bradykinin potentiating peptide (BPPb, Pyr-GLPPRPKIPP), pdb codes 4APH
379 and 4APJ, respectively (Masuyer et al., 2012). In the structure of ACE bound to BPPb, the binding of
380 BPPb caused the elimination of the zinc ion, and significant conformation changes compared with
381 the enzyme in complex with AngII (Masuyer et al., 2012), which is interesting for docking studies as
382 two different conformations of the active site are considered.

383 The initial docking of peptides into ACE was done using the induced fit docking protocol of the
384 Schrödinger suite (Schrödinger, 2011; Sherman, et al., 2006a; Sherman et al., 2006b). This protocol
385 allows considering side chain flexibility of residues around 5 Å from the ligand. Various parameters
386 as IFD score, and visual inspection were used to select those conformations that best match the
387 enzyme binding-site. The IFD score is an estimation of the binding energy for each output pose, it is

388 computed based on the GlideScore and a small fraction of the Prime energy. Interestingly, a better
389 complementarity was obtained when the docking was carried out using the protein conformation
390 derived from ACE-BPPb complex. However, these models have a limitation, as only partially side
391 chain flexibility has been considered. To overcome this limitation and to allow the flexibility of the
392 whole protein, molecular dynamics studies were then applied to refine these ACE-peptide complex
393 models. Explicit-solvent molecular dynamics simulations were carried out using Amber, the ff12SB
394 force field and the TIP3 water model (Pearlman et al., 1995; Case et al., 2012). After the first
395 equilibration steps, in which positional restrains were applied, the flexibility of both the enzyme and
396 the peptide was allowed, in order to optimize the interactions between them. The analysis of the final
397 models revealed a series of key residues of ACE for the interaction with lentil peptides, several of
398 them also involved in the binding of AngII or BPPb.

399 Molecular docking studies have revealed that Ct-1, Ct-3 and Ct-4 occupied the same location in the
400 active site of ACE (Figure 1A) as AngII or bradikinin, with a similar orientation of the N- and C-
401 terminus, and sharing important residues for the interaction, principally with the S2' subsite. These
402 heptapeptides showed more interactions with the enzyme toward the C-terminal region. In particular,
403 there are several hydrogen bonds that are maintained through the dynamic studies, involving the C-
404 terminal carboxylic group of peptides Ct-1, Ct-3 and Ct-4, and the sidechains of two ACE residues,
405 namely Tyr520 and Lys511, besides this group in derivatives Ct-3 and Ct-4 also showed a hydrogen
406 bond with the NH₂ group of Gln281 side-chain. These three ACE residues are also involved in the
407 binding of AngII and BPPb. It is also interesting to note that the amino terminus of these three
408 peptides is involved in hydrogen bonds, with the side-chain of Glu403 for peptides Ct-3 and Ct-4, a
409 residue also involved in the binding of BPPb, whereas for peptide Ct-1 there is a hydrogen bond to
410 Asp358 side-chain and backbone CO, a residue implicated in the interaction with AngII. Besides,
411 each peptide is further stabilized through additional hydrogen bonds and hydrophobic interactions, as
412 detailed below.

413 Peptide Ct-1 is anchored to the enzyme through a network of hydrogen bonds in which almost all
414 amino acids are implicated (Figure 1B), as the ones that engaged the backbone CO of Pro1 and Leu4,
415 the former interacts through two hydrogen bonds with Asp358 of ACE, the latter with His513 side
416 chain. The side-chain of the third residue (Gln3) forms hydrogen bonds with the side-chains of ACE
417 Asn66 and Trp357. Additionally, Ser6 of peptide Ct-1 is involved in two hydrogen bonds with the
418 Glu411 side-chain. Next residue, Phe7, is stabilized by a π - π -interaction with Tyr523, and is within a
419 binding pocket formed by two other aromatic residues, Phe457 and Phe527 (Figure 1B). A special
420 mention deserves the two interactions of the NH₂ group of Gln5 side-chain with ACE, namely a
421 hydrogen bond with the carbonyl of Ala354, and a rare NH- π interaction with His353 sidechain
422 (Steiner & Kowllner, 2001). Gln5 residue is substituted in peptide 5 with Glu, and this replacement
423 leads to a considerable decrease in ACE inhibition. Thus, the mentioned interactions of the Gln5 side
424 chain might account for the higher affinity of peptide Ct-1 compared with Ct-5.

425 Peptide Ct-3 was the one with the higher affinity for ACE (this C-heptapeptide sequence is also
426 shared for peptide 7). There are fewer interactions in the N-terminal region over the C-terminal one,
427 among them, hydrogen bonds involving Leu1 amino group and the side-chain of Glu403, and several
428 hydrophobic interactions, thus, Leu1 of Ct-3 is close to Phe391, and Pro2 to Trp59, Tyr62 and
429 Tyr360 (Figure 1C). On the contrary, the C-terminal tripeptide fragment (RYL) is strongly anchored
430 to the enzyme through ionic pairs, π - π interactions and hydrogen bonds. Thus, it is observed a good
431 electrostatic complementarity between the binding pocket and the charged residues, in particular salt
432 bridges are formed between the Arg5 side-chain and the COO terminal group of Ct-3 and Glu143
433 and Lys511 side-chains of ACE, respectively. Leu7 residue binds in the S2' subsite through a series
434 of hydrogen bonds, involving its C-terminal group and the sidechains of Lys511, Tyr520 and Gln281
435 of ACE (Figure 1C). The hydrogen bond with Lys511 is frequently bifurcated. Tyr6 residue of Ct-3
436 shows a hydrogen bond between the backbone NH and the CO of Ala354 of ACE. This hydrogen
437 bond was also observed in the 4APH X-ray structure, involving a backbone NH of AngII. The side-

438 chain of Tyr6 is also stabilized through hydrophobic interactions, in particular, π - π interactions with
439 Tyr523, and it is surrounded by Phe457 and Phe527. Besides, Arg5 residue forms a bifurcated
440 hydrogen bond between the guanidinium group and Phe512 of ACE, a hydrogen bond with Glu143,
441 and likely with Ser516 (Figure 1C). Additionally, it is observed a stacking between the guanidinium
442 group and Phe512. It has been described that the geometry of this type of stacking allows a cation- π
443 interaction, as well as additional non electrostatic van der Waals and π - π interactions. On the whole,
444 there is a network of hydrogen bonds that stabilized the C-terminal tripeptide RYL, reinforced by
445 stacking with aromatic rings. This strong interaction is in agreement with the fact that this sequence
446 has been found in a series of peptides with capacity to inhibit ACE (BIOPEP database). It is
447 interesting to note that the N-terminal sequence of peptide Ct-6 RLL, differs from that of peptide Ct-
448 3 in that the residue of Tyr6 has been replaced by Leu6, and this change resulted in considerable
449 lower inhibition. Therefore, one factor that may influence this lower inhibition is the loss of the
450 stacking between the aromatic ring of Tyr523 of ACE and the Tyr6 of peptide Ct-3.

451 Peptide Ct-4 is anchored to ACE through the already mentioned hydrogen bonds involving the C-
452 and N-terminus residues. Besides, the main chain of peptide Ct-4 is stabilized by further hydrogen
453 bonds, as the one between the CO of Leu5 and ACE His513 side-chain, as well as between Val4 NH
454 and the backbone CO of Ala356 ACE (Figure 1D). These residues of ACE are also involved in
455 hydrogen bonds with AngII and BPPb. This peptide is additionally stabilized through a series of
456 hydrophobic contacts. Thus, Phe1 and Leu2 are surrounded by a cluster of aromatic residues, namely
457 Trp59, Tyr62 and Tyr360. Pro3 residue is situated very similar to the Pro7 of BPPb and is also
458 stabilized by hydrophobic interactions with Trp357 of the enzyme, while Leu5 is located close to
459 Phe512. Finally, Leu6 is also within a binding pocket delimited by tyrosines 520 and 523, and
460 phenylalanines 457 and 527 (Figure 1D).

461 During the last years, it has become apparent that ACE is involved in other physiological processes
462 outside blood regulation and that its C- and N-domain are involved in divergent biological processes

(Bernstein et al., 2013; Harrison & Acharya, 2014; Masuyer et al., 2012). The C-domain has been shown to process angiotensin I more efficiently than the N-domain, while angiotensin 1–7, β -amyloid 1–42 or peptide AcSDKP are better hydrolyzed by the N-domain, finally both hydrolyze bradykinin with similar efficiency. Thus, there is an interest in C-domain selective inhibitors, in order to provide control of blood pressure, and at the same time to allow an active N-domain. Several inhibitors with selectivity for the C- or N-domain have been described, and their selectivity has been explained on the base of key residues that differ between the two domains (Harrison & Acharya, 2014). It is worth mentioning that Glu403 residue of C-ACE is replaced by an Arg (Arg381) in N-ACE, a residue involved in the binding of Ct-3 and Ct-4, which might suggested a certain selectivity of these two peptides for the C-domain over the N-domain.

4. Conclusions

In this study, we have demonstrated that fragments from lentil storage proteins vicilin, convicilin and legumin show dual antioxidant and ACE inhibitory activities. Gastrointestinal digestion of lentil-derived peptides improved markedly their dual activity suggesting their promising potential to exert antihypertensive effect *in vivo*. Nevertheless, additional preclinical and clinical studies should be carried out to assess their *in vivo* antihypertensive effect. Electronic, hydrophobic and steric properties of amino acid residues in the C-terminal region were important structural features of peptides for high antioxidant properties, while positively charged amino acids and hydrophobic amino acid residues are determinant for high ACE inhibitory potency. In addition, molecular docking studies have revealed that C-terminal heptapeptide residues of lentil-derived peptides interact by hydrogen bonds with three ACE residues of the catalytic site, also involved in the binding of AngII and BPPb (Tyr520 and Lys511 and Gln281). This characterization of lentil peptides helps clarify the relationship between peptide structure and dual antioxidant/antihypertensive activity and supports the application of lentil protein hydrolysates as ingredients for development of functional foods.

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615 Figure captions

616 Fig. 1. Close-up view of (A) The superposition of the models of the C-domain of ACE in complex
617 with peptide Ct-1 (C atoms in purple), Ct-3 (C atoms in cyan) or Ct-4 (C atoms in green), peptides in
618 stick representation. (B-D) The model of the ACE C-domain in complex with peptide Ct-1 (B), Ct-3
619 (C) and Ct-4 (D) showing their binding modes. For clarity only polar hydrogens are shown. The
620 hydrogen bonds are depicted as grey dashed lines.

Table 1

Most abundant peptides identified in 3 kDa permeates of lentil hydrolysates produced by enzymatic hydrolysis with Savinase® 16 L.

Code	Sequence	Parent proteins	MH+ (Da)
1	DLAIPVNRPGQLQSF	Vicilin Allergen Len c 1.0101	1654.89
2	LLSGTQNPQSFLSGF	Allergen Len c 1.0101 Allergen Len c 1.0102	1595.81
3	NSLTLPIRLYL	Legumin type B beta chain	1302.78
4	TLEPNSVFLPVLLH	Vicilin-like antimicrobial peptides 2-3	1578.89
5	DLAIPVNNPGQLESF	Allergen Len c 1.0102	1613.82
6	RFQTIYNENGHIRLL	Allergen Len c 1.0101 Allergen Len c 1.0102	2003.04
7	LVNEGKGNLELVGF	Convicilin	1488.81
8	EITPEKNPQLQDLDFVN	Allergen Len c 1.0101 Allergen Len c 1.0102 Vicilin	2113.08

Table 2

Antioxidant and ACE inhibitory (ACEI) activities of complete and C-terminal heptapeptide sequence of identified peptides in lentil protein hydrolysates

Code	Number of aa	Peptide	ORAC ^{2,4}	ACEI ^{3,4}
Complete sequence				
1	15	DLAIPVNRPGQLQSF	0.015±0.001aB	>500
2	15	LLSGTQNQPSFLSGF	0.013±0.000aA	119.75±3.04bB
3	11	NSLTLPIRLYL	1.432±0.068cA	77.14±2.31aB
4	14	TLEPNSVFLPVLLH	0.139±0.005bB	117.81±8.92bB
5	15	DLAIPVNNPGQLESF	0.004±0.000aA	>300
6	16	RFQTIYENENGHIRLL	Nm ⁵	>250
7	14	LVNEGKGNLELVGF	0.009±0.000aB	>500
8	17	EITPEKNPQLQDLDFVN	0.049±0.002a	>500
C-terminal heptapeptide sequence				
Ct 1	7	PGQLQSF	0.006±0.000aA	51.75±1.03ab
Ct 2	7	PSFLSGF	0.015±0.001aB	74.03±0.42bA
Ct 3	7	LPILRYL	2.521±0.207bB	14.99±0.07aA
Ct 4	7	FLPVLLH	0.086±0.012aA	25.66±0.36abA
Ct 5	7	PGQLESF	0.011±0.001aB	377.30±29.13d
Ct 6	7	NGHIRLL	0.035±0.006a	244.85±6.43c
Ct 7	7	NLELVGF	0.006±0.000aA	342.40±49.21d
Ct 8	7	DLDFVN	nm	nm

²ORAC of peptides expressed as Trolox equivalents (μmol Trolox eq./μmol pure peptide)

³ACEI of peptides expressed as IC₅₀ (concentration of peptide in μM needed to inhibit ACE activity by 50%)

⁴Lowercase letters indicate statistical differences among complete or C-terminal heptapeptide sequences ($p < 0.05$, Duncan test). Uppercase letters indicate differences between each C-terminal heptapeptide and its parent sequence ($p < 0.05$, Duncan test).

⁵nm, not measured

Table 3

Functional stability of peptides identified in lentil protein hydrolysates to simulated gastrointestinal digestion (SGD)

Code	Sequence	ORAC ^{1,3}		ACEI ^{2,3}	
		Before SGD ⁴	After SGD	Before SGD	After SGD
1	DLAIPVNRPGQLQSF	0.015±0.001aA	10.87±0.89aB	>500	11.4±0.78a
2	LLSGTQNQPSFLSGF	0.013±0.000aA	14.29±1.65cB	119.75±3.04bB	21.25±1.17cA
3	NSLTLPIRLYL	1.432±0.068cA	9.66±0.02aB	77.14±2.31aB	20.475±0.52cA
4	TLEPNSVFLPVLLH	0.139±0.005bA	12.59±1.42bB	117.81±8.92bB	16.045±0.95bA
5	DLAIPVNNPGQLESF	0.004±0.000aA	9.65±1.24aB	>300	>100
6	RFQTIYENENGHIRLL	nm ⁵	9.85±1.20a	>250	74.54±0.52d
7	LVNEGKGNLELVGF	0.009±0.000aA	16.61±1.59dB	>500	>100
8	EITPEKNPQLQDLDFVN	0.049±0.002aA	10.28±1.29aB	>500	>100

¹ORAC of peptides expressed as $\mu\text{mol Trolox eq.}/\mu\text{mol pure peptide}$ ²ACEI of peptides expressed as IC_{50} (concentration of peptide in μM needed to inhibit ACE activity by 50%)³Lowercase letters indicate statistical differences among values within a column ($p<0.05$, Duncan test). Uppercase letters indicate differences between treatments for each biological activity ($p<0.05$, Duncan test).⁴SGD, simulated gastrointestinal digestion⁵nm, not measured

Table 4List of peptides and amino acids generated by simulated *in silico* gastrointestinal digestion of lentil peptides

Code	Sequence	Position of cleavage site	Peptides	Amino acids	Reported bioactivity
1	DLAIPVNRPGQLQSF	1, 2, 9, 11, 12, 14, 15	AIPVNRP, QS, GQ	L, F, D	QS: dipeptidyl peptidase IV inhibitor
2	LLSGTQNQPSFLSGF	1, 2, 11, 12, 14, 15	SGTQNQPSF, SG	L, F	SG: ACE inhibitor
3	NSLTLPIRLYL	2, 3, 5, 8, 9, 10, 11	PIL, NS, TL	L, R, Y, L	PIL: ACE inhibitory peptide from egg white (YAEERYPIL) TL: C-terminal sequence of antioxidant peptide PSLVGRPPVGKLTL, dipeptidyl peptidase IV inhibitor
4	TLEPNSVFLPVLLH	1, 2, 3, 7, 8, 9, 12, 13, 14	PNSV, PVL	T, F, L, H, E	PVL: C-terminal sequence of ACE inhibitory and antioxidant peptides from β -CN (YQQPVL and VKVLLLEHPVL, respectively)
5	DLAIPVNNPGQLESF	1, 2, 11, 12, 13, 14, 15	AIPVNNPGQ	D, L, F, S, E	
6	RFQTIYENENGHIRLL	1, 2, 5, 6, 7, 9, 12, 14, 15, 16	QTI, NE, NGH, IR	R, F, Y, L, E	IR: C-terminal sequence of ACE inhibitory peptide AVNPIR, antioxidant peptide, dipeptidyl peptidase IV inhibitor
7	LVNEGKGNLELVGF	1, 4, 6, 9, 10, 11, 13, 14	VNE, GK, GNL, VG	L, E, F	VG: dipeptidyl peptidase IV inhibitor
8	EITPEKNPQLQDLDFVN	1, 5, 6, 10, 11, 12, 13, 15, 16, 18	ITPE, NPQL, DI, VN	L, F, E, K, Q, D	QD: dipeptidyl peptidase IV inhibitor DI: C-terminal dipeptide of ACE inhibitor and antioxidant peptides (KRQKYDI and VGDI, respectively) VN: dipeptidyl peptidase IV inhibitor

Simulated *in silico* gastrointestinal digestion of peptides 1-8 was performed using the Peptide Cutter tool from ExPASy (http://web.expasy.org/peptide_cutter/) and the selection of chymotrypsin-low specificity (C-term to [FYWML], not before P), pepsin (pH>2), and trypsin.

Fig. 1