



High-pressure improves enzymatic proteolysis and the release of peptides with angiotensin I converting enzyme inhibitory and antioxidant activities from lentil proteins



P. Garcia-Mora, E. Peñas, J. Frias, R. Gomez, C. Martinez-Villaluenga *

Institute of Food Science, Technology and Nutrition, C/ Juan de la Cierva 3, 28006 Madrid, Spain

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ABSTRACT

Angiotensin I converting enzyme (ACE) inhibitory and antioxidant peptides are receiving attention due to their beneficial effects in the prevention/treatment of hypertension. The objective was to explore the effect of high hydrostatic pressure (HP) on proteolysis by different proteases and the release of bioactive peptides from lentil proteins. Pressurisation (100–300 MPa) enhanced the hydrolytic efficiency of Protamex, Savinase and Corolase 7089 compared to Alcalase. Proteolysis at 300 MPa led to a complete degradation of lentil proteins and increased peptide (<3 kDa) concentration by all enzymes. Proteolysis at 300 MPa by Savinase gave rise to lentil hydrolysates (S300) with the highest ACE-inhibitory and antioxidant activities that were retained upon *in vitro* gastrointestinal digestion. The peptides responsible for the multifunctional properties of S300 hydrolysate were identified as different fragments from storage proteins and the allergen Len c 1. These results support the potential of HP as a technology for the cost-effective production of bioactive peptides from lentil proteins during enzymatic proteolysis.

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1. Introduction

Cardiovascular diseases (CVD) are a worldwide health problem that represents a significant burden not only on the medical care system but also on the long-term quality of life of human population. Elevated blood pressure is one of the major independent risk factors for cardiovascular disease (Erdmann, Cheung, & Schroeder, 2008). Angiotensin converting enzyme (ACE, EC 3.4.15.1) is one of the main regulators of blood pressure; thus, inhibition of this enzyme is considered as one of the strategies for the treatment of hypertension (Hong et al., 2008). Moreover, recent evidences have found oxidative stress as one important factor underlying hypertension (Bagatini et al., 2011). Excessive amounts of reactive oxygen species affects cellular functions (Ray, Huang, & Tsuji, 2012), reduces the bioavailability of endothelial nitric oxide (Toeroek, 2008), and enhances low density lipoprotein oxidation in the vascular system (Mattson, 2009). Therefore, oxidative stress has emerged as an additional therapeutic target for prevention or treatment of hypertension.

The existing evidence continues to confirm the importance of a healthy diet and lifestyle to prevent the incidence of CVD and their related risk factors (World Health Organization, 2009). Functional foods containing bioactive proteins and peptides have

demonstrated clinical improvements in CVD risk factors (Cam & de Mejia, 2012). Biologically active peptides released from food proteins by gastrointestinal digestion or food processing possess multiple bioactive properties (antihypertensive, antioxidant, anti-inflammatory and hypocholesterolemic) and their consumption may play a significant role in promoting cardiovascular health (Hernández-Ledesma, García-Nebot, Fernández-Tomé, Amigo, & Recio, 2014). For these reasons, there has been a strong interest in the production of functional hydrolysates containing bioactive peptides for their application in functional foods that promote cardiovascular health. So far, many of the research performed on functional hydrolysates has used animal food-derived proteins (milk, egg, fish, meat) as raw materials for the production of bioactive peptides (Erdmann et al., 2008; Martínez-Maqueda, Miralles, Recio, & Hernández-Ledesma, 2012). Alternatively to animal proteins, legumes represent an economical and environmentally sustainable protein source for food industry that has begun to be investigated for the same purposes. Lentil contains approximately 28% protein on a dry weight basis (Roy, Boye, & Simpson, 2010) and its global production ranks fourth among pulse crops (Food, 2012). The enzymatic hydrolysis of lentil proteins have resulted in the production of hydrolysates with ACE-inhibitory activity and bile salts binding activity (Barbana, Boucher, & Boye, 2011; Barbana & Boye, 2011). Moreover, our research group has recently identified specific fragments from legumin, vicilin and convicilin with amino acid sequences contributing to the antioxidant and ACE-inhibitory

* Corresponding author. Tel.: +34 912587601; fax: +34 915644853.

E-mail address: c.m.villaluenga@csic.es (C. Martinez-Villaluenga).

activity of lentil hydrolysates (García-Mora, Peñas, Frias, & Martínez-Villaluenga, 2014).

High hydrostatic pressure (HP) processing has experienced a huge growth in the last 20 years to become an industrial reality (Norton & Sun, 2008). HP offers the food industry applications in food preservation and creation of novel foods, textures and tastes. Besides these major applications, HP has also been used in combination with protease treatments for the production of hypoallergenic (López-Expósito et al., 2008; Peñas, Restani, et al., 2006b) and functional hydrolysates containing bioactive peptides (Hoppe, Jung, Patnaik, & Zeece, 2013; Zhang, Jiang, Miao, Mu, & Li, 2012). Previous studies have demonstrated that enzymatic proteolysis under high pressure conditions enhances the protein susceptibility to digestion (Chicón, Belloque, Recio, & López-Fandiño, 2006; Quirós, Chicón, Recio, & López-Fandiño, 2007) which makes this technology worthy of consideration to increase hydrolytic products yields and to reduce reaction time and production costs. The extent of proteolysis achieved during HP treatment greatly depends on the protein system (type of protein, pH and ionic strength of the medium), type of protease, applied pressure level and duration time of pressure treatment (Belloque, Chicón, & López-Fandiño, 2007; Chicón et al., 2006; López-Expósito et al., 2008; Peñas, Snel, Floris, Préstamo, & Gomez, 2006c). The literature about the application of high-pressure assisted proteolysis in the production of bioactive peptides from legume proteins has been largely unexplored. There is only one recent study showing that high pressure pre-treatment of chickpea proteins reduces hydrolysis time and enhance the formation of antioxidant peptides in the hydrolysates (Zhang et al., 2012).

The aim of this work was to study whether high pressure enhances the proteolytic efficiency of several proteases and to evaluate the impact of this treatment on the release of peptides with ACE-inhibitory and antioxidant activity.

2. Materials and methods

2.1. Materials

Lentil seeds (*Lens culinaris* var. Castellana) were provided by Semillas Iglesias S. A. (Salamanca, Spain) and stored in polyethylene bins at 4 °C. Commercial food-grade enzymes Alcalase® 2.4L FG (2.4 AU/g), Savinase® (16 KNU/g) and Protamex® (1.5 AU/g), were kindly provided by Novozymes (Bagsvaerd, Denmark). Corolase 7089 was provided by AB Enzymes GmbH (Darmstadt, Germany). The tripeptide Abz-Gly-Phe(NO₂)-Pro was purchased from Cymit-Química (Barcelona, Spain). All other chemicals were purchased from Sigma (Barcelona, Spain) unless otherwise specified.

2.2. Preparation of lentil protein concentrates

Whole lentil seeds were ground using a centrifugal mill (Moulinex, Allençon, France) and passed through a 60-mesh sieve with 0.5 mm pore size. Flours were stored at –20 °C before use. The lentil protein concentrates were prepared using alkaline extraction as described in García-Mora et al. (2014). Briefly, lentil flour was suspended in water (solid-to-solvent ratio 1:10, w/v) and the pH was adjusted to 8. The suspension was stirred in an orbital shaker (Infors, Switzerland) at 20 °C for 1 h and then vacuum-filtered using a filter funnel (100–160 µm nominal pore size) to remove solids. Lastly, filtrates were freeze-dried and stored under vacuum in plastic bags at –20 °C until further analysis.

2.3. High pressure (HP)-assisted proteolysis

Freeze-dried lentil protein concentrates were suspended in deionised water (2%, w/v), equilibrated at 40 °C and the pH

adjusted to 8 with 0.1 M NaOH. Enzymatic proteolysis was carried out following the method of Cupp-Enyard (2008) using an enzyme to substrate ratio of 0.1 Anson Units/mg of soluble protein at 40 °C and pH 8. Anson Units are defined as the micromoles of tyrosine released from the substrate per minute. HP treatment was performed using a Stansted Fluid Power Iso-lab 900 High Pressure Food Processor (Model FPG7100:9/2C, Stansted Fluid Power Ltd., Harlow, Essex, UK) with 3 L capacity, maximum pressure of 900 MPa, and a potential maximum temperature of 100 °C. Four packed samples were introduced into the pressure unit filled with water, then treated at pressures of 100, 200, 300, 400 or 500 MPa. Pressure was increased at a rate of 600 MPa/min and maintained at the desired pressure for a holding time of 15 min; the decompression time was less than 4 s. The temperature of the pressure unit vessel was thermostatically controlled at 40 °C throughout all the treatments. The temperature of the pressure unit level was thermostatically controlled by a computer program, being constantly monitored and recorded during the process. The lentil protein concentrate was also pressurised at pH 8 without enzyme for 15 min. All HP treatments were performed in duplicate. Control hydrolysis experiments were carried out at atmospheric pressure (0.1 MPa) at 40 °C for 15 min. Enzymatic reactions were stopped by heating at 80 °C for 15 min. Finally, hydrolysates were centrifuged at 14,000 rpm, at 10 °C for 10 min, freeze-dried and stored at –20 °C until use. HP-assisted proteolysis and hydrolysis at atmospheric pressure were performed in triplicate.

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

SDS-PAGE analysis of the protein hydrolysates was performed on NuPAGE® Novex 4–12% Bis-Tris Gels using the XCell-sure lock Mini-Cell (Invitrogen, Madrid, Spain). Electrophoresis was carried out at 200 V, and the running and sample buffers used were NuPAGE® MES-SDS, and NuPAGE® LDS (Invitrogen), respectively. Runs were carried out under non-reducing conditions in which 2-mercaptoethanol was omitted in the denaturing buffer. Electrophoretic bands were stained with SimplyBlue SafeStain (Invitrogen), followed by destaining in deionised water. The molecular weight of poly- and oligopeptides was determined by comparison with the molecular weight marker solution Mark 12™ (Invitrogen) containing myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66.3 kDa), glutamic dehydrogenase (55.4 kDa), lactate dehydrogenase (36.5 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6.0 kDa), insulin B chain (3.5 kDa) and insulin A chain (2.5 kDa).

2.5. Determination of soluble protein and the content of peptides <3 kDa

Soluble protein content was measured in pressurised and non-pressurised lentil protein concentrates and hydrolysates at pH 8 by DC protein assay (Biorad) using bovine serum albumin (BSA) as standard. For peptide concentration, hydrolysates were submitted to ultrafiltration through membranes of 3 kDa pore size (Millipore Corporation, Billerica, MA, USA) and permeates were analysed by the DC protein assay (Biorad) using BSA as standard.

2.6. Determination of ACE-inhibitory activity

Protein hydrolysates and their controls were passed through a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) before ECA inhibitory assay to eliminate non-protein compounds. The retained compounds were eluted with 60% acetonitrile containing 0.1% TFA in water. ACE-inhibitory activity of samples was further measured

in duplicate at a protein concentration of 0.5 mg/mL. The fluorescence-based protocol of Sentandreu and Toldrá (2006) was used. The generated fluorescence was read every minute for 30 min at emission and excitation wavelengths of 355 and 405 nm, respectively, in a microplate fluorometer (Biotek, Winooski, VT, USA). IC_{50} values expressed in protein concentration (mg/mL) were calculated for the most active hydrolysates. IC_{50} was determined by dose–response curves in which the range of protein concentration was distributed in a logarithmic scale and using the non-linear regression sigmoidal curve fit function in GraphPad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).

2.7. Determination of oxygen radical absorbance capacity (ORAC)

Samples were passed through a Sep-Pak C18 cartridge (Waters, Milford, MA, USA) and retained compounds were eluted with 60% acetonitrile containing 0.1% TFA in water. The antioxidant capacity of samples was measured in duplicate by fluorescence using the ORAC method as described previously (Torino et al., 2013). Results were expressed as μ mol Trolox equivalents (TE)/g hydrolysate.

2.8. Identification of bioactive peptides by MALDI/TOF

The proteomics analysis was carried out by matrix-assisted laser desorption/ionisation time of flight tandem mass spectrometry (MALDI TOF/TOF) at the Proteomics Facility UCM-FPCM, a member of ProteoRed-ISCI network. Hydrolysates having the highest ACE-inhibitory and antioxidant activity were selected for peptide identification. Sample (1 μ L) was spotted onto a MALDI target plate and allowed to air-dry at room temperature. Then, 0.4 μ L of a 3 mg/mL of α -cyano-4-hydroxy-transcinnamic acid matrix (Sigma) in 50% acetonitrile were added to the dried peptide digest spots and allowed again to air-dry at room temperature. Analyses were performed in a 4800 Plus MALDI TOF/TOF Analyzer mass spectrometer (Applied Biosystems, Framingham, MA) operating in positive reflector mode, with an accelerating voltage of 20 kV. All mass spectra were calibrated internally using peptides from the auto digestion of trypsin. The analysis by MALDI TOF mass spectrometry produces peptide mass fingerprints and the peptides observed with a Signal to Noise greater than 20 can be collated and represented as a list of monoisotopic molecular weights. Proteins ambiguously identified by peptide mass fingerprints were subjected to MS/MS sequencing analyses using the 4800 Plus MALDI TOF/TOF Analyzer (Applied Biosystems, Framingham, MA). The most intense multiply charged ions were selected for collision-induced dissociation. Fragment-ion spectra (MS/MS spectra) were acquired in the ion reflector mode over the m/z range 50–2500 and were sequenced by using the PepSeq de novo sequencing algorithm (Micromass).

For protein identification, NCBI nr taxonomy Viridiplantae (1530236 sequences) and a home-made *L. culinaris* database with the Uniprot entries was searched using MASCOT 2.3 (matrix-science.com) through the Global Protein Server v3.6 from Applied Biosystems. The presence of the identified peptides in lentil proteins was confirmed using the BLAST tool (<http://www.blast.ncbi.nlm.nih.gov/Blast.cgi>). The search parameters were the following: (i) peptide mass tolerance 80 ppm; (ii) MS–MS fragments tolerance 0.3 Da; and (iii) oxidised methionine as variable modification.

2.9. In vitro gastrointestinal digestion

Lentil hydrolysates produced by selected protease type and hydrolysis time were further subjected to simulated gastrointestinal digestion by sequential hydrolysis using pepsin and pancreatin without the presence of phosphatidylcholine according to Moreno,

Mellon, Wickham, Bottrill, and Mills (2005). Digestions were stopped by heating samples in boiling water for 10 min. Samples were stored at -20°C and then freeze-dried. Protein content was determined for each sample using the DC Protein Assay (Biorad) and BSA as standard.

2.10. Statistical analysis

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

3. Results and discussion

3.1. Effect of pressurisation on lentil protein solubility at alkaline pH

Protein solubility is a critical factor in the hydrolytic yield of proteins that may be negatively affected by pressure treatment, therefore, soluble protein concentration of HP-treated protein concentrates at different pressure levels was firstly measured. Pressurised protein concentrates at 100, 200 and 300 MPa showed similar soluble protein content as control (8.21 ± 0.44 mg protein/mL), while higher pressures of 400 and 500 MPa significantly reduced the soluble protein content (5.9 ± 0.72 mg protein/mL, respectively) ($P < 0.05$). Our result agrees with previous studies showing a decrease in the solubility of lupin, green pea, soybean and chickpea 7S and 11S globulins at pressure levels of 400–450 MPa or higher (Angioloni & Collar, 2013; Chapleau & De Lamballerie-Anton, 2003). In contrast, HP-treated globulins of red kidney bean showed enhanced solubility with increasing pressure levels (Yin, Tang, Wen, Yang, & Li, 2008). This difference may be attributed to differences of protein type, nature and conformational stability between legume proteins. The reduced protein solubility observed at 400 and 500 MPa for lentil proteins could be explained by the formation of insoluble protein aggregates. Pressure-induced protein aggregates have been formed in kidney bean protein isolates by a rupture of hydrophobic interactions in the protein core that lead to exposure of the buried hydrophobic groups and sulphhydryl (SH) groups to the protein surface resulting in a gradual unfolding of vicilins structure (Yin et al., 2008). Upon pressure release, the exposed hydrophobic and free sulphhydryl groups can be involved in new hydrophobic interactions and disulphide bonds, leading to vicilin aggregation of the pressure-denatured protein (Yin et al., 2008). Based on these results, pressure levels from 100 to 300 MPa were used to study the effect of pressurisation on enzymatic hydrolysis of lentil proteins.

3.2. Effect of pressurisation on enzymatic hydrolysis of lentil proteins and release of peptides

Fig. 1 (panel A) presents the SDS–PAGE profile under non-reducing conditions of controls and samples hydrolysed by Alcalase, Protamex, Savinase and Corolase 7089 at 0.1, 100, 200 and 300 MPa. Electrophoretic profile of lentil protein concentrate (control) showed intense bands with apparent molecular masses (MM) from 14.4 to 95 kDa. The major bands found in lentil concentrate had estimated MM of 50 and 65 kDa, which correspond to subunits of 7S globulins such as vicilin (48 kDa) and convicilin (63 kDa), respectively, according to UniProt database (<http://www.uniprot.org/>). Other bands with lower MM of 40, 20 and <15 kDa were considered to belong to 11S acidic subunit, 11S basic subunit and a mixture of γ -vicilin and albumin polypeptides, respectively (<http://www.uniprot.org/>). Pressurisation at 100, 200 and 300 MPa did not

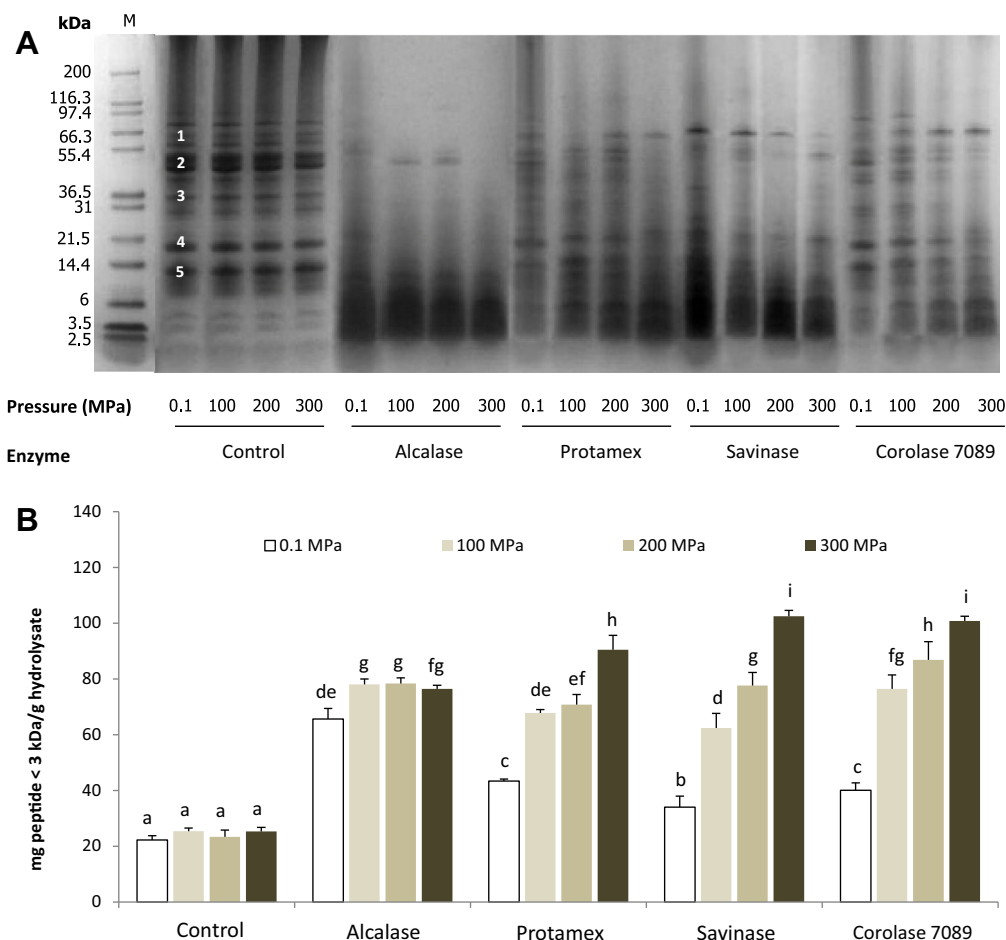


Fig. 1. SDS–PAGE profile (A) and peptide (<3 kDa) content (B) of lentil proteins hydrolysed by commercial proteases at different pressures (0.1, 100, 200, 300 MPa) for 15 min. M: molecular weight marker (Mark 12™, invitrogen); 1: Convicilin; 2: Vicilin; 3: 11S acidic subunit; 4: 11S basic subunit; 5: γ -vicilin, albumin. Means with different letters are significantly different ($P \leq 0.05$, Duncan test). Error bars represent the standard deviation of the mean.

alter the electrophoretic protein profile of lentil protein concentrates (Fig. 1 panel A). Unlike what has been reported for other proteins such as β -lactoglobulin and ovalbumin, the absence of new bands in the SDS–PAGE profile confirms that HP treatment of lentil proteins did not give rise to dissociation of globulin subunits or formation of disulphide-linked oligomers (Chicón, Belloque, Alonso, & López-Fandiño, 2008; Chicón et al., 2006; Quirós et al., 2007).

Protein bands corresponding to 7S and 11S globulins were absent or drastically reduced following Alcalase and Savinase incubation, respectively (Fig. 1, panel A) and, consequently, a greater intensity was observed for low molecular weight bands <10 kDa. In contrast, Protamex and Corolase 7089 protein digestion resulted in a slightly decreased intensity of the major protein bands giving rise to hydrolysates composed of polypeptides with a wide range of MM from 70 to 14 kDa. These observations are consistent with our previous results, showing that complete proteolysis of lentil proteins was achieved after 1 and 3 h of hydrolysis at atmospheric pressure by Alcalase and Savinase, respectively (Garcia-Mora et al., 2014). In contrast, Protamex and Corolase 7089 treatment for 6 h was inefficient to remove all intact protein (Garcia-Mora et al., 2014). Moreover, enzymatic hydrolysis of lentil proteins at 0.1 MPa by all enzymes increased significantly the content of peptides <3 kDa ($P \leq 0.05$) compared to control (Fig. 1B). Hydrolysates produced at atmospheric pressure by Alcalase showed the highest content of peptides <3 kDa (65.6 mg/g hydrolysate) followed by Protamex (43.4 mg/g hydrolysate), Corolase 7089 (40.1 mg/g hydrolysate) and Savinase (34.0 mg/g hydrolysate).

As compared to the proteolysis at atmospheric pressure, proteolysis under HP led to qualitative differences in the hydrolysis pattern observed by SDS–PAGE, except for Alcalase (Fig. 1 panel A). Alcalase digestion of lentil proteins under HP slightly increased the concentration of small peptides at pressures from 100 to 300 MPa (1.19-fold) compared to hydrolysis at atmospheric pressure (Fig. 1B). This observation is consistent with a previous study showing that Alcalase activity increased slightly at low pressure levels (100–300 MPa) (Zhang et al., 2012). In contrast, pressurisation above 200 MPa considerably increased lentil proteins susceptibility to enzymatic hydrolysis by Savinase, Protamex and Corolase (Fig. 1, panel A). In these cases, the extent of proteolysis increased with pressure level up to 300 MPa. 7S and 11S globulins were more extensively or completely hydrolysed under HP (200–300) after 15 min. HP-assisted proteolysis at 300 MPa enhanced drastically the intensity of protein fragments <6 kDa (Fig. 1A) and the concentration of peptides <3 kDa (Fig. 1B) compared to at lower pressure levels. These results agree with previous works on HP-assisted proteolysis of ovalbumin by trypsin, chymotrypsin and pepsin that brought about increased levels of the proteolysis products (Quirós et al., 2007). These results are in agreement with previous reports showing that pressurisation during enzyme treatment enhances hydrolysis of soy (Peñas, Préstamo, Baeza, Martínez-Molero, & Gomez, 2006a; Peñas, Restani, et al., 2006b; Peñas, Snel, et al., 2006c), chickpea (Zhang et al., 2012), kidney bean (Yin et al., 2008, egg white (Hoppe et al., 2013) and milk proteins (Peñas, Préstamo, et al., 2006a) by different enzymes.

Enhancement of protein susceptibility to proteolysis under pressure can be explained by the exposure of new cleavage sites on the substrate through several pressure-induced phenomena such as protein unfolding and enhancement in structural flexibility of the substrate that expose new cleavage sites (Belloque et al., 2007). HP can also enhance enzyme activity and/or the substrate-enzyme interaction (Stapelfeldt, Petersen, Kristiansen, Qvist, & Skibsted, 1996). Changes in the three-dimensional structure of enzyme could also affect its active site resulting in an increased activity or a change in the substrate specificity as have been suggested (Claeys, Indrawati, Van Loey, & Hendrickx, 2003). On the contrary, pressure levels above 300 MPa may cause a loss of their activity or a complete inactivation as it have been reported for trypsin or Alcalase (Chicón et al., 2006; Yin et al., 2008; Zhang et al., 2012). In the case of pressure-sensitive enzymes a different approach in the production of protein hydrolysates is the application of HP before enzymatic hydrolysis. Peñas, Préstamo, et al. (2006a), Peñas, Restani, et al. (2006b), Peñas, Snel, et al. (2006c) found a more extensive proteolysis in hydrolysates when cows milk whey was pressurised before Corolase 7089 treatment instead of simultaneously to proteolysis.

3.3. Effect of high pressure (HP) on ACE-inhibitory activity of lentil hydrolysates

Lentil protein concentrates showed a weak ACE inhibitory activity (20%) that was significantly increased upon enzymatic digestion treatment ($P \leq 0.05$) regardless of the enzyme used (Table 1). Alcalase and Savinase digestion at atmospheric pressure gave rise to higher values of ACE inhibition (57% and 46%, respectively) than Protamex and Corolase 7086 (36% and 34%, respectively). These results were also observed in our previous work, although higher ACE inhibition values were found in lentil hydrolysates submitted to longer incubation times up to 2–3 h depending on the protease type (García-Mora et al., 2014).

The ACE inhibitory activity of lentil protein concentrates (0.1 MPa) remained similar after pressurisation (100–300 MPa). However, HP treatment during lentil proteolysis affected differently the ACE-inhibitory activity of hydrolysates produced by different enzymes (Table 1). Enzymatic hydrolysis with Alcalase at 100 MPa caused a reduction in the ACE inhibitory activity of hydrolysates compared to controls at atmospheric pressure

($P < 0.05$). Moreover, higher pressures (200 and 300 MPa) brought about a higher decrease in the ACE-inhibitory activity of hydrolysates produced from Alcalase. Even though pressurisation slightly enhanced proteolytic degradation by Alcalase and accumulation of small peptides <3 kDa (Fig. 1) there was not a direct relationship with the ACE inhibitory activity of hydrolysates. These results might be due to the release of amino acid sequences with lower ACE inhibitory activity. In contrast, enzymatic proteolysis by Protamex, Savinase or Corolase 7089 at high pressure (100–300 MPa) significantly improved the ACE-inhibitory activity of hydrolysates ($P \leq 0.05$) compared to their controls at 0.1 MPa. Protamex digestion at 200 MPa gave rise to lentil hydrolysates exhibiting a higher ACE inhibition (65.3%) than hydrolysates obtained at 100 and 300 MPa. ACE-inhibitory activity of hydrolysates produced by Savinase and Corolase 7089 under high pressure was noticeably improved with increasing pressure levels up to 300 MPa. Finally, combined Savinase and Corolase 7089 treatments with pressures of 300 MPa resulted in the highest values of ACE-inhibitory activity (69.5% and 70.8% ACE inhibition, respectively). Comparing these results from our previous study, longer time of hydrolysis at atmospheric pressure was needed to achieve similar ACE inhibitory values to those found in lentil hydrolysates produced from Savinase under pressure (García-Mora et al., 2014). Moreover, lentil proteolysis by Corolase 7089 at 0.1 MPa during 6 h brought about hydrolysates with a significantly lower ACE inhibitory activity (28–50% inhibition) (García-Mora et al., 2014) than those obtained at 300 MPa. Our results indicated that HP promoted the release of bioactive sequences likely due to the higher accessibility of enzymes to the substrate and the exposure of new target residues. The protease specificity and the optimum degree of hydrolysis also play a very important role in the release and accumulation of peptides with ACE-inhibitory activity (Quirós et al., 2007). In agreement with our results previous studies higher amounts of antihypertensive peptides were produced after 1 h of ovalbumin hydrolysis under 200–400 MPa than after 8 h of hydrolysis at atmospheric pressure (Quirós et al., 2007).

3.4. Effect of high pressure (HP)-assisted proteolysis on antioxidant activity of lentil hydrolysates

Protein digestion at atmospheric pressure for 15 min did not change the initial ORAC values (242.2 $\mu\text{g TE/g}$ protein concentrate)

Table 1

ACE-inhibitory activity of lentil protein hydrolysates produced by HP-assisted proteolysis by commercial proteases at different pressures.

Pressure (MPa)	ACE inhibition (%)				
	Control	Alcalase	Protamex	Savinase	Corolase 7089
0.1	20.34 $\pm$ 1.23 ^A	57.24 $\pm$ 3.92 ^D	36.6 $\pm$ 0.06 ^B	45.99 $\pm$ 1.38 ^C	34.58 $\pm$ 1.92 ^B
100	22.24 $\pm$ 1.89 ^A	55.73 $\pm$ 2.92 ^B	51.24 $\pm$ 5.33 ^B	54.27 $\pm$ 0.04 ^B	52.51 $\pm$ 1.62 ^B
200	25.85 $\pm$ 4.81 ^A	50.68 $\pm$ 5.29 ^B	65.29 $\pm$ 6.19 ^C	52.52 $\pm$ 2.50 ^B	61.08 $\pm$ 1.36 ^C
300	20.49 $\pm$ 3.23 ^A	40.80 $\pm$ 2.25 ^A	57.76 $\pm$ 0.12 ^C	69.46 $\pm$ 0.09 ^C	70.77 $\pm$ 0.20 ^C

Data indicate mean value $\pm$ standard deviation of two independent experiments. Means with different lowercase letters within a column are significantly different ($P < 0.05$, Duncañs test). Means with different uppercase letters within a row are significantly different ($P < 0.05$, Duncañs test). Control: sample with no enzyme.

Table 2

Oxygen radical absorbance capacity (ORAC) of lentil protein hydrolysates produced by HP-assisted proteolysis by commercial proteases at different pressures.

Pressure (MPa)	ORAC ($\mu\text{moles TE/g}$)				
	Control	Alcalase	Protamex	Savinase	Corolase 7089
0.1	242.16 $\pm$ 19.74 ^B	251.48 $\pm$ 10.95 ^A	245.16 $\pm$ 15.61 ^A	224.42 $\pm$ 13.18 ^A	248.79 $\pm$ 24.03 ^A
100	231.93 $\pm$ 17.35 ^{A_{ab}}	278.79 $\pm$ 13.51 ^B	274.30 $\pm$ 14.36 ^B	271.12 $\pm$ 14.35 ^B	313.88 $\pm$ 17.85 ^C
200	214.17 $\pm$ 21.99 ^A	225.75 $\pm$ 18.02 ^A	265.40 $\pm$ 20.07 ^{B_c}	416.37 $\pm$ 35.09 ^D	330.32 $\pm$ 19.74 ^C
300	215.37 $\pm$ 10.28 ^A	225.68 $\pm$ 14.15 ^A	232.10 $\pm$ 22.45 ^A	403.86 $\pm$ 21.10 ^C	360.99 $\pm$ 8.31 ^B

Data indicate mean value $\pm$ standard deviation of two independent experiments. Means with different lowercase letters within a column are significantly different ($P < 0.05$, Duncañs test). Means with different uppercase letters within a row are significantly different ($P < 0.05$, Duncañs test). Control: sample with no enzyme.

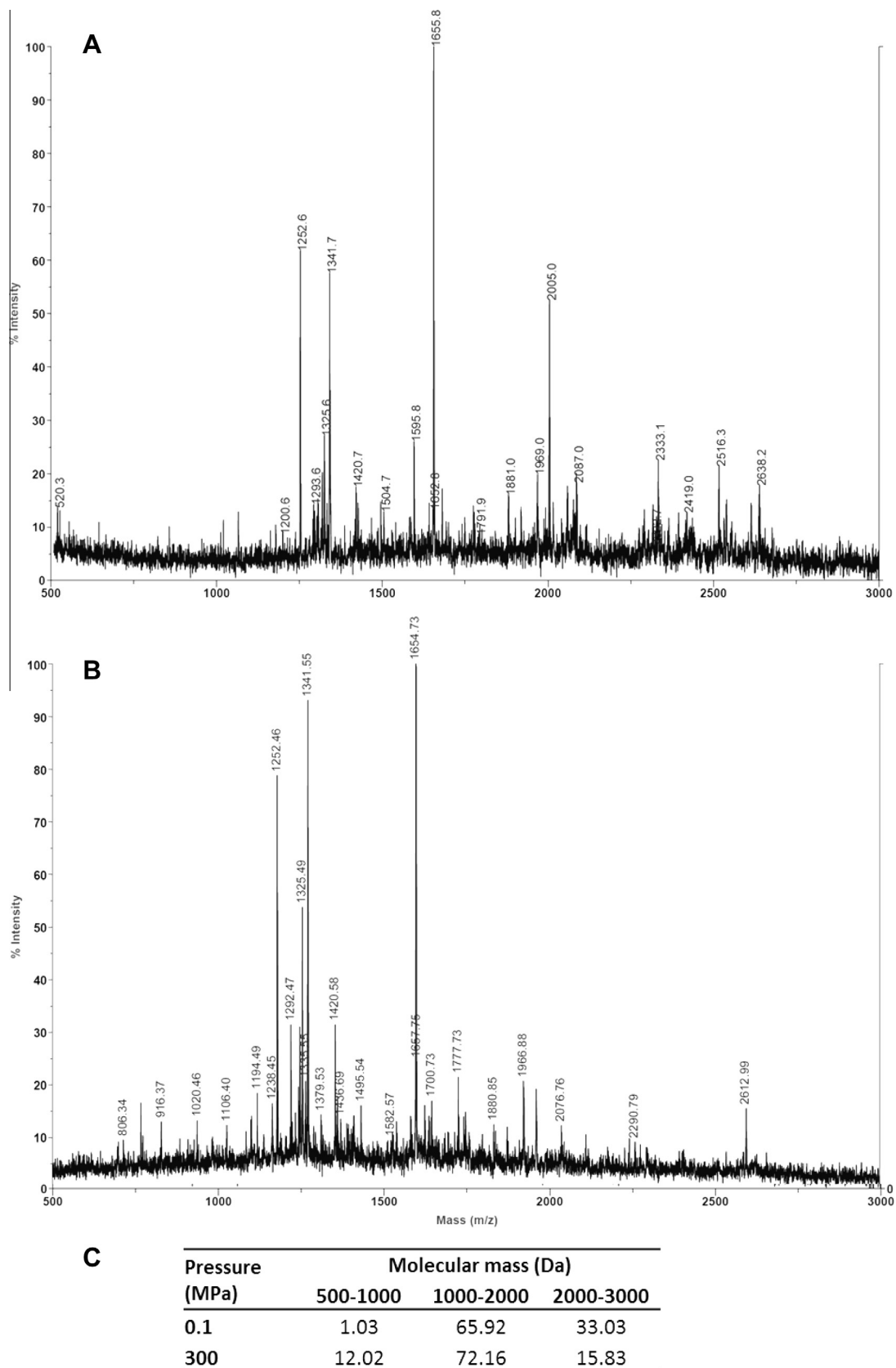


Fig. 2. Representative peptide mass fingerprints of 3 kDa permeates of hydrolysates derived from Savinase digestion at atmospheric pressure (A) and 300 MPa (B) for 15 min. Percentage distribution of peptide masses (C) found in lentil hydrolysates produced by Savinase at 0.1 and 300 MPa.

observed in non-hydrolysed lentil proteins (Table 2). The combined treatment of pressure at 100 MPa and enzymatic proteolysis resulted in hydrolysates with higher ORAC values than those obtained at 0.1 MPa ($P < 0.05$). Higher pressure levels of 200 and 300 MPa applied during enzymatic proteolysis had a different impact on the antioxidant activity of hydrolysates depending on the protease used (Table 2). ORAC values of hydrolysates produced by Alcalase at 200 and 300 MPa were significantly lower than the ones produced at 100 MPa ($P < 0.05$). A similar result was found when proteolysis was performed by Protamex at 300 MPa. In contrast, Savinase digestion at higher pressure levels (200 and 300 MPa) resulted in increased antioxidant activity compared to hydrolysates obtained at 100 MPa ($P < 0.05$). In this case, ORAC values reached a two-fold increase versus control (S0.1 hydrolysates). On the other hand, similar ORAC values were observed for hydrolysates produced by Corolase 7089 at the different pressure levels tested (100, 200 and 300 MPa). Finally, it is worth noting that proteolysis conducted by Savinase at 200 and 300 MPa resulted in the highest ORAC values (416.4 and 403.9 $\mu\text{g TE/g}$ hydrolysate). Similarly to our results, Zhang et al. (2012) reported an increased antiradical activity of chickpea hydrolysates obtained by Alcalase treatment at 100–200 MPa for 10 min that was related to increased amounts of low molecular weight peptides. However, Alcalase treatment of chickpea protein isolate at 300 MPa did not improve the antiradical activity of hydrolysates due to protein aggregation and enzyme deactivation. In controversy with previous works, in our study there was not a direct relationship between antioxidant activity of hydrolysates and the amount of low molecular weight peptides (Carrasco-Castilla et al., 2012). Similarly to the ACE inhibitory activity, the specificity of the protease used to release antioxidant peptides and the optimum degree of proteolysis that accumulates bioactive peptides is what determines the antioxidant activity of hydrolysates.

3.5. Effect of pressurisation on peptides recovered in the 3 kDa permeates of selected lentil hydrolysates

Hydrolysate produced by Savinase at 300 MPa was selected for further characterisation of the peptide fraction due to its higher concentration of peptides <3 kDa, ACE-inhibitory and antioxidant activities. Small peptide fractions are mostly the major contributors to the ACE-inhibitory and antioxidant activity of protein hydrolysates. For this reason, peptide characterisation was performed in the peptide fraction <3 kDa obtained by ultrafiltration

through a 3 kDa membrane. Fig. 2 presents representative MALDI TOF peptide mass fingerprints of 3 kDa permeate from hydrolysates produced by Savinase at 0.1 (Panel A) and 300 MPa (Panel B). Major differences between MALDI spectra were found for ions with molecular masses from 2000 to 3000 Da. Ions at m/z of 2333.1, 25516.3, 2636.2 were not present in hydrolysates produced at 300 MPa. HP affected to the distribution of peptide molecular masses in the hydrolysate that was characterised by increased percentage of peptides <2000 Da and a lower percentage of peptides >2000 Da (Fig. 2 panel C). The five intense signals at m/z 1252.46, 1325.49, 1341.55, 1420.58 and 1654.73 observed in the hydrolysates obtained by Savinase digestion at 300 MPa (Fig. 2; panel B) were identified as DLPVLRWLKL, SRSDQDNPFIF, REQIEELRL, DLAI PVNRPGQLQ, DLAI PVNRPGQLQSF, respectively (Table 3). These peptides have not been previously identified and corresponded to different fragments of the main storage lentil proteins (Legumin, Vicilin and Convicilin) and allergens (Len c 1.0101 and 1.0102) (Table 3). Other less intense signals were mostly identified as fragments of allergens Len c 1.0101 and 1.0102. We previously identified the peptide DLPVLRWLKL along with other peptide sequences by LC–MS/MS in lentil hydrolysates produced by Savinase proteolysis at atmospheric pressure for 2 h (Garcia-Mora et al., 2014). The difference in the peptide pattern between the hydrolysates produced by Savinase at atmospheric pressure for 2 h and 300 MPa for 15 min could be attributed to the different degree of hydrolysis achieved in the hydrolysates. The predicted biological activities found in the amino acid sequence of these peptides were ACE-inhibitory (bolded sequences) and antioxidant activities (underlined sequences). Moreover, these peptides had residues such as alanine (A), valine (V), leucine (L), and phenylalanine (F) at the C-terminus, all of them considered as target residues for the ACE catalytic site (Cheung, Wang, Ondetti, Sabo, & Cushman, 1980). Regarding structure–activity relationship of antioxidant peptides, several reports have related the antiradical activity with the prevalence of hydrophobic amino acids such as Ala (A), Pro (P), Val (V), Ile (I), Leu (L), Phe (F), Trp (W), and Tyr (Y) which may act as proton donors. These amino acids were present in the identified peptides of the present study (Contreras, Hernandez-Ledesma, Amigo, Martin-Alvarez, & Recio, 2011; Elias et al., 2006). Specifically, Tyr (Y) and Trp (W) have been reported as the main responsible for the antioxidant activity of peptides in the ORAC method (Elias, Kellerby, & Decker, 2008). His (H) was also found within the amino acid sequence of some of the identified peptides. This amino acid has been commonly associated with antioxidant

Table 3

Peptides identified by MALDI TOF/TOF in the 3 kDa permeate of lentil hydrolysates produced by HP-assisted proteolysis using Savinase at 300 MPa for 15 min.

Observed mass	Calculated mass	Fragment	Peptide sequence	Intensity (%)
1106.40	1105.22	Legumin A1 f(424–432) Legumin A2 f(431–439)	SDRFTYVAF	12.16
1194.49	1194.31	Allergen Len c 1.0101/1.0102 f(21–30)	ENENGHIRLL	18.24
1238.45	1238.32	Vicilin type C f(29–38) Allergen Len c 1.0101 f(2–11)	RSDQDNPFIF	16.29
1252.46	1252.57	Legumin A f(370–379) Legumin A2 f(373–382)	DLPVLRWLKL	78.65
1325.49	1325.40	Vicilin type C f(28–38) Allergen Len c 1.0101/1.0102 f(1–11)	SRSDQDNPFIF	53.62
1341.55	1341.53	Convicilin f(352–361)	REQIEELRL	92.95
1420.58	1420.63	Allergen Len c 1.0101 f(121–133)	DLAI PVNRPGQLQ	31.29
1426.63	1426.64	Allergen Len c 1.0101/1.0102 f(123–135)	AIPVNRPGQLQSF	15.49
1654.73	1654.89	Allergen Len c 1.0101/1.0102 f(122–135)	DLAI PVNRPGQLQSF	100
1700.73	1699.88	Allergen Len c 1.0101/1.0102 f(17–30)	QTIYENENGHIRLL	16.77
1777.73	1778.76	Allergen Len c 1.0101/1.0102 f(311–325)	DEEEGQEEETTKQVQ	21.32
1880.85	1881.21	Allergen Len c 1.0101/1.0102 f(122–137)	DLAI PVNRPGQLQSFLL	12.28
2612.99	2613.81	Allergen Len c 1.0101/1.0102 f(159–179)	FNTEYEEIEKVLLEEQEQSKQ	15.36

Bolded and underlined sequence regions correspond to reported ACE-inhibitory and antioxidant activity, respectively, in BIOPEP (<http://www.uwm.edu.pl/biochemia/index.php/pl/biopep>).

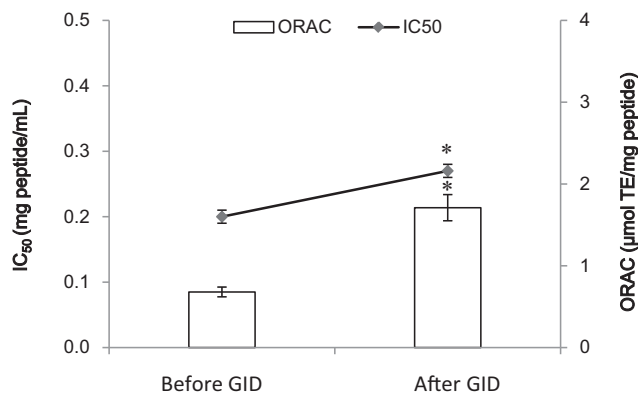


Fig. 3. Effect of gastrointestinal digestion (GID) on the ACE-inhibitory and antioxidant activity of 3 kDa permeates of hydrolysates produced by Savinase at 300 MPa. Lines represent ACE-inhibitory activity and columns show antioxidant activity before and after *in vitro* gastrointestinal digestion. *Statistically different ($P < 0.05$) from non-digested control.

activity due to its hydrogen-donating and radical-trapping imidazole ring (Elias et al., 2008), therefore, peptides containing His are likely to contribute to the antioxidant activity observed in lentil hydrolysates. The identification of all these peptide sequences supported the multifunctional properties of lentil hydrolysates produced by Savinase treatment at 300 MPa as mentioned above.

3.6. Effect of gastrointestinal digestion on ACE-inhibitory and antioxidant activities of selected lentil hydrolysates

Gastrointestinal enzymes may cause structural degradation of food-derived peptides consequently affecting their biological activity. Therefore, to study how gastrointestinal digestion could affect the biological activity of peptides present in the hydrolysate obtained by Savinase at 300 MPa, 3 kDa permeates were sequentially digested with pepsin and pancreatin under simulated gastrointestinal conditions. Fig. 3 shows ACE-inhibitory (expressed as IC₅₀) and antioxidant activities (expressed as μmol TE/mg peptide) of 3 kDa permeate of the selected hydrolysate before and after *in vitro* gastrointestinal digestion. *In vitro* gastrointestinal digestion caused a slight loss of ACE-inhibitory activity as evidenced by higher IC₅₀ values (0.27 mg peptide/mL) compared to non-digested control (0.20 mg peptide/mL) ($P \leq 0.05$). On the contrary, simulated gastrointestinal digestion resulted in a 2-fold increase in the antioxidant activity of the 3 kDa permeate which reached an ORAC value of 1.71 μmol TE/mg peptide. This observation could be explained by the release of new fragments by gastrointestinal enzymes with higher antioxidant capacity.

4. Conclusions

In comparison to atmospheric pressure, 300 MPa enhanced the proteolytic efficiency of four food-grade proteases (Alcalase, Protamex, Savinase and Corolase 7089) giving rise to a higher degradation of the major lentil storage proteins and a greater accumulation of peptides <3 kDa. Increased ACE-inhibitory activity was observed in hydrolysates when HP assisted the proteolysis of the different enzymes, with exception of Alcalase. Moreover, there is also a potential benefit to be gained by application of this technology to increase the antioxidant capacity of lentil hydrolysates through a selection of the appropriate protease and pressure level. In this study, proteolysis at 300 MPa by Savinase gave rise to lentil hydrolysates with the highest ACE inhibitory and antioxidant activities in a relatively short time (15 min). In addition, the biological activity of these hydrolysates was highly retained (ACE inhibitory activity)

or improved (antioxidant activity) upon *in vitro* gastrointestinal digestion. The identification of several peptides in these hydrolysates containing bioactive amino acid sequences in their structure support the potential of HP treatment as a technology to efficiently release bioactive peptides from lentil proteins.

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