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Savinase the most suitable enzyme for releasing peptides from lentil (*Lens culinaris* var. Castellana) protein concentrates with multifunctional properties

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ABSTRACT

The aim of this study was to produce multifunctional hydrolyzates from lentil protein concentrates. Four different proteases (Alcalase, Savinase, Protamex and Corolase 7089) and different hydrolysis times were evaluated for their degree and pattern of proteolysis, angiotensin I converting enzyme (ACE)-inhibitory and antioxidant activities. Alcalase and Savinase showed the highest proteolytic effectiveness ($P \leq 0.05$) which resulted in higher yield of peptides. The hydrolysate produced by Savinase after 2 h of hydrolysis (S2) displayed the highest ACE-inhibitory (IC_{50}=0.18 mg/mL) and antioxidant activity (1.22 μmol Trolox equivalents/mg protein). Subsequent RP-HPLC-MS/MS analysis of 3kDa permeates of S2 showed 32 peptides mainly derived from convicilin, vicilin and legumin containing amino acid sequences which makes them potential contributors to ACE-inhibitory and antioxidant activities detected. The ACE-inhibitory and antioxidant activity of S2 were significantly improved after *in vitro* gastrointestinal digestion ($P \leq 0.05$). Multifunctional hydrolysates could encourage value-added utilization of lentil proteins for the formulation of functional foods and nutraceuticals.

Keywords: Lentil protein hydrolysates, alkaline proteases, antioxidant peptides, ACE inhibitory peptides

INTRODUCTION

Hypertension is a public health problem associated with cardiovascular complications affecting 25% adult population worldwide.[1] Moreover, the increasing number of individuals with the metabolic syndrome is contributing to the rising prevalence of hypertension. In view to its high prevalence, pharmacotherapy, diet and lifestyle modifications are applied for treatment of hypertension. Inhibition of angiotensin I-converting enzyme (ACE, E.C. 3.4.15.1) has been used as a target therapy for treatment of hypertension.[2] This enzyme plays a crucial role in the renin-angiotensin and kinin-kalicrein systems regulating blood pressure.[3] Inhibition of ACE reduces the concentration of angiotensin II, a vasoconstrictor octapeptide, while it increases levels of the vasodilator peptide bradikinin which results in the lowering of blood pressure.[2]

A recent study has shown the increased participation of reactive oxygen species and reduced antioxidant enzymatic defense in the pathogenesis of hypertension.[4] Oxidative stress affect several cellular signaling cascades and cellular functions[5] reducing the bioavailability of nitric oxide, a vasodilatory molecule involved in the smooth muscle relaxation in the cardiovascular system.[6] In addition, oxidation of membrane lipids brings about lipid peroxidation products, which damage proteins in the cardiovascular system.[7] Therefore, amelioration of oxidative stress in addition to inhibition of ACE provides a multitarget therapy that may improve the clinical efficacy of pharmacological treatments for the management of hypertension and other cardiovascular disorders.

Because of the adverse side effects of the synthetic drugs, functional foods containing natural compounds are alternative strategies to drug therapy.[8] Food-derived bioactive peptides with more than one bioactivity have multifunctional properties that can potentially be useful in targeting the multiple pathophysiological conditions underlying hypertension.[9] Much research has been focused on the generation of peptides derived from animal and plant food proteins for the management of

hypertension.[10] To date, milk is the main source of ACE inhibitory peptides, in addition to egg, meat and fish.[11] However, plant sources have gained less attention in this respect.

Lentil is a traditional pulse crop providing economic benefits in addition to the benefits derived from crop rotation, nitrogen fixation, and sustainable agriculture.[12] Lentils are the staple food in many countries providing an inexpensive source of protein in the diet (20–30%), however, they are largely underexploited for new emerging applications in the functional foods and nutraceutical areas. Previous studies have shown the potential of lentil proteins as source of ACE inhibitory peptides.[13, 14] In contrast, no studies have been conducted to produce multifunctional hydrolyzates from lentil proteins exhibiting both ACE-inhibitory and antioxidant activities so far, which would open up new possibilities for value-added applications of lentil.

Bioactive peptides may be produced by enzymatic hydrolysis of food proteins with enzymes from microbial, plant or gastrointestinal origin. Several enzymes have been used to produce lentil hydrolyzates with bioactive properties such as Alcalase+Flavourzyme, bromelain, papain and gastrointestinal enzymes.[13,14] These studies have shown that ACE-inhibitory activity of lentil hydrolyzates strongly depends on protease specificity. Moreover, the release of bioactive peptides and consequently, the biological activity of protein hydrolyzates can be affected by operational conditions such as hydrolysis time.[15] The objective of this work was to produce multifunctional lentil hydrolysates with ACE-inhibitory and antioxidant peptides. The effect of protease type and hydrolysis time on the proteolytic pattern, ACE-inhibitory and antioxidant activities of lentil hydrolysates was also studied. This information is critical for the development of value-added products, particularly as ingredient/s for the functional food and nutraceutical markets.

MATERIALS AND METHODS

Materials. Lentil seeds (*Lens culinaris* var. Castellana) were provided by Semillas Iglesias S. A. (Salamanca, Spain) and stored in polyethylene bins at 4 ºC. Commercial food-grade enzymes Alcalase® 2.4L FG, Savinase® and Protamex®, were kindly provided by Novozymes (Bagsvaerd, Denmark). Alcalase and Savinase are alkaline serine proteases from *Bacillus licheniformis* and *Bacillus* sp., respectively. Protamex is a *Bacillus* protease complex consisting of sustilisin and neutral protease (E.C. 3.4.24.28). Corolase 7089 is a fungal neutral protease provided by AB Enzymes GmbH (Darmstadt, Germany). Tripeptide Abz-Gly-Phe(NO_2)-Pro was purchased from Cymit-Quimica (Barcelona, Spain). All other chemicals were purchased from Sigma-Aldrich Quimica (Madrid, Spain) unless otherwise specified.

Preparation of lentil protein concentrates. Whole lentil seeds were ground using a coffee mill (Moulinex, Allençon, France) and passed through a 60-mesh sieve and 0.5 mm pore size. The lentil powders were stored at -20 °C before use. Total protein concentration in lentil flour was determined as total nitrogen multiplied by 6.25. Total nitrogen of lentil flours was analyzed in duplicate using a LECO TRUMAC apparatus (LECO Corp., St. Joseph, MI, USA).

Lentil protein concentrates were prepared using alkaline extraction. Briefly, lentil flour was suspended in water (solid-to-solvent ratio 1:10, w/v) and the pH value was adjusted to 8. The suspension was stirred in an orbital shaker (Infors, Switzerland) at 20 ºC 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 and dark conditions in plastic bags at -20 ºC until further analysis. The soluble protein concentration in the filtrates was determined by the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA) following the manufacturer´s protocol. Bovine serum albumin (BSA) was used as standard at a concentration range from 0 to 1 mg/mL.

Enzymatic proteolysis. Freeze-dried lentil protein concentrates were suspended in deionized water (2%, w/v), equilibrated at 40 ºC and the pH value adjusted to 8 with 0.1 M NaOH. Enzymatic proteolysis was carried out using an enzyme to substrate ratio (E/S) of 0.1 AU/mg of soluble protein at 40 ºC and pH 8. Aliquots were withdrawn at 0, 1, 2, 3, 4, 5, and 6 h from reaction mixtures and heated at 80 ºC for 15 min for enzyme inactivation. Finally, hydrolyzates were centrifuged at 23,430 *g*, at 10 ºC for 10 min, freeze-dried and stored at -20 ºC until use. Protein concentration was determined by the DC protein assay (Bio-Rad). The samples were coded for protease type (A = Alcalase; S = Savinase; P = Protamex and C =Corolase 7089) followed by hydrolysis time (1, 2, 3, 4, 5 and 6). For example, S2 hydrolyzates are those produced from lentil concentrate hydrolyzed by Savinase for 2 h. Hydrolysis were performed at least in triplicate for each enzyme.

Degree of hydrolysis (DH). The DH was calculated by determination of free amino groups by reaction with 2,4,6-Trinitrobenzenesulfonic acid according to Adler-Nissen.[16] Total number of amino groups in lentil protein concentrate was determined after acid hydrolysis in 6 N HCl at 110 ºC for 24 h in vials sealed under nitrogen. Analyses were performed in duplicate. Degree of hydrolysis was calculated using the following equation:

$$DH\ (\%) = 100 \times [(AN_2 - AN_1) / N_{pb}]$$

Where AN_1 is the amino nitrogen content in the protein substrate before hydrolysis (mg tyrosine equivalents/g protein), AN_2 is the amino nitrogen content in the free protein in the protein substrate after hydrolysis (mg tyrosine equivalents/g protein) and N_{pb} is the total amino groups in the protein substrate (mg tyrosine equivalents/g protein) as determined with 6 N HCl at 110 ºC for 24 h.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE analysis of the protein hydrolyzates 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.

NuPAGE® MES-SDS and NuPAGE® LDS were used as running and sample buffers (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 deionized water. The molecular weight of poly- and oligopeptides was determined by comparison with the molecular weight marker solution Mark 12^{TM} (Invitrogen).

ACE-inhibitory activity. ACE-inhibitory activity of samples was measured in duplicate following the fluorescence-based protocol of Sentandreu and Toldrá.[17] 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 Synergy HT (Biotek, Winooski, VT, USA). IC_{50} values expressed in protein concentration (mg/mL) were calculated for the most active hydrolyzates. IC_{50} was determined by dose–response curves in which the range of protein concentration (0-0.5 mg/mL) 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). Protein concentration of 3 kDa permeates was measured by DC protein assay (Biorad).

Oxygen radical absorbance capacity (ORAC). ORAC of samples was measured in duplicate by fluorescence as described previously.[18] Results were expressed as μmol Trolox equivalents (TE)/ mg protein (μmol TE/mg protein).

Proteomic analyses. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis were performed for the most active hydrolyzates. Peptide mass fingerprint was performed in a Voyager-DE PRO mass spectrometer (Applied Biosystems, Foster City, CA) equipped with a pulsed 337 nm nitrogen laser (1 ns pulse width and 3 Hz frequency) which was operated in the reflectror mode for positive ions. Parameters of the instrument were as follows: an acceleration voltage of 20 kV, an

158 extraction voltage of ions generated by the laser desorption were introduced into a time of flight analyser
159 (1.3 m flight path) with an acceleration voltage of 20 kV, 76% grid voltage, 0.001% ion guide wire
160 voltage, and a delayed extraction time of 400 ns. The samples were mixed with the matrix at a ratio of
161 1:5 (v/v), and 1 µL of this solution was spotted onto a flat stainless-steel sample plate and dried in air.
162 Mass spectra were obtained over de m/z range 500-4000. External mass calibration was applied using
163 the monoisotopic [M + H]+ values of des-Arg^1 Bradykinin, Angiotensin I, Insuline, Glu^1-Fibrino-
164 peptide B, adrenocorticotropic hormone fragments 1-17, 18-39, and 7-38.

165 For peptide identification, 10 µg of the most active hydrolysate were desalted using a reverse phase
166 (PorosR2©) chromatography with 80% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA). Later, the
167 peptides were dried and resuspended with 10 µL 0.1% TFA. An aliquot of 5 µl of resuspended sample
168 were injected in the Linear Trap Quadrupole (LTQ) Orbitrap Velos (Thermo Scientific). Peptides were
169 loaded onto a C18-A1 ASY-Column 2 cm precolumn (Thermo Scientific) and then eluted onto a
170 Biosphere C18 column (C18, inner diameter 75 µm, 15 cm long, 3 µm particle size) (NanoSeparations)
171 at a flow rate of 250 nL/min on a nanoEasy high-performance liquid chromatography (Proxeon) coupled
172 to a nanoelectrospay ion source (Proxeon).

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

Proteome Discoverer 1.4.288 (Thermo) with MASCOT 2.3 was used to search in NCBI database, taxonomy Viridiplantae (1530236 sequences sequences) and a home-made database with the *Lens culinaris* entries in UniProt (336 sequences, 75114 residues). Database search parameters used were the following: peptide tolerance, 10 ppm; fragment ion tolerance, 0.8 Da; none enzyme and variable modification, methionine oxidation. In all peptide identification, the probability scores were greater than the score fixed by Mascot as significant with a p-value minor than 0.05. The automatic decoy database search function of Protein Discover was enabled to allow estimation of false discovery rate (FDR). Sequence identification with reported biological activity was compared with bioactive peptides sequences submitted to the BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

***In vitro* gastrointestinal digestion.** Lentil hydrolyzates produced by selected protease type and hydrolysis time were further subjected to simulated gastrointestinal digestion by sequential hydrolysis using pepsin and pancreatin.[19] Digestions were stopped by heating samples in boiling water for 10 min. Samples were stored at -20 ºC and then freeze-dried. Protein content was determined for each sample using the DC Protein Assay (Biorad).

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.

RESULTS AND DISCUSSION

Protein content of lentil flour (27.4±0.1% on dry basis) was within the range of reported values for lentil flours.[20] Alkaline extraction at pH 8 led to 80% extraction of total protein. Thus, protein content of lentil var. Castellana protein concentrate was 75.54±0.2% on dry basis which was comparable

204 to previous data for red and green lentil protein concentrates (78.2±0.2, 79.1±0.3%).[21] The obtained
205 lentil protein concentrate was hydrolyzed by different enzymes (Alcalase, Savinase, Protamex and
206 Corolase 7089) for screening the most effective one hydrolyzing lentil proteins and releasing ACE-
207 inhibitory and antioxidant peptides.

208 **Proteolytic pattern of hydrolysis.** To evaluate the hydrolysis efficiency, the degree of hydrolysis
209 (DH) value is a widely used criterion as it is highly related to the hydrolytic process yield. Figure 1
210 shows the effect of protease type and hydrolysis time in the DH of lentil proteins. DH values increased
211 gradually reaching a plateau at 5 h for Alcalase, Savinase and Protamex (23%, 15%, 11%) and 4 h for
212 Corolase 7089 (10%). The significant differences ($P \leq 0.05$) found among DH values of lentil
213 hydrolyzates may be attributed to different enzyme specificity.[22] Subtilisins have a broad specificity
214 which explains its higher proteolytic efficiency compared to Protamex and Corolase 7089. Our results
215 agree with previous studies outstanding the ability of Alcalase to produce protein hydrolyzates with
216 higher DH than other enzymes.[23] Maximum DH observed in the present study for Alcalase hydrolyzates
217 (23%) is comparable to those reported in earlier studies for other legume proteins.[24, 25] In contrast, the
218 degree of hydrolysis of cowpea and bean proteins by Alcalase was found significantly different from
219 lentil proteins (35.7 and 14%, respectively) .[26, 27] These differences in the susceptibility of lentil proteins
220 to hydrolysis might be attributed to their particular structure.[28] Enzymatic treatments with sequential
221 addition of Alcalase (1 h) and Flavourzyme (1.5 h) have also been used to increase the proteolytic
222 efficiency in the production of lentil hydrolyzates.[29] In this case, high DH values were obtained (58 and
223 64% for red and green lentil proteins, respectively); however, extensive proteolysis is not recommended
224 because peptides generated may produce bitterness and worsen important technological properties
225 required for particular food industry applications.

Figure 2 shows the electrophoretic profiles of lentil hydrolyzates produced by Alcalase (Panel A), Savinase (Panel B), Protamex (Panel C) and Corolase 7089 (Panel D). Non-hydrolysed protein concentrate (Figure 2 panels A, B, C and D; Lane 2) showed nine 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 probably correspond to subunits of vicilin (48 kDa) and convicilin (63 kDa), respectively.[30] 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.[31] The electrophoretic profile of protein concentrates from lentil var. Castellana was similar to that reported for green and red lentil concentrates.[13] Major polypeptides found in lentil protein concentrate were readily hydrolysed by Alcalase to small peptides < 10 kDa after 1 h (Figure 2A; Lane 3). Longer hydrolysis time from 4 to 6 h induced further breakdown of peptide bonds and formation of much smaller fractions at MM < 6 kDa (Figure 2A; Lanes 6-8). Our results agree with previous studies in which Alcalase digestion was efficient in producing small peptides with MM below 10 kDa from chickpea,[24] and bean[26, 31] proteins. In addition, sequential use of Alcalase and Flavorzyme to produce peptides from lentil proteins have been proven more effective than the use of gastrointestinal enzymes, papain and bromelain.[13] Similarly to Alcalase, protein digestion by Savinase was also efficient. A sharply reduction of most polypeptides (30, 40, 50, 55 and 65 kDa) present in lentil protein concentrate was observed after 1 h of hydrolysis appearing of new bands under 14 kDa (Figure 2B; Lane 3). As the hydrolysis progressed, polypeptides with estimated MM at 50, 55, 65 kDa were completely degraded (Figure 2B; Lanes 4-8). In addition, the reduction of intensity of bands with MM <14 kDa suggested further degradation of the peptides into much smaller fragments. In contrast, Protamex and Corolase 7089 digestions were not as efficient as Alcalase and Savinase digestions in consistency with DH values (Figure 2C and 2D). Protamex digestion completely degraded high- and medium-molecular weight

polypeptides (estimated MM at 30, 37, 60, 67, 90 kDa) within 1 h (Figure 2C; Lane 3). As consequence, the appearance of some bands between 30 and 20 kDa and < 10 kDa was observed. Further treatment with Protamex resulted in slight changes in the digest profile such as the disappearance of some bands with apparent MM between 30 and 20 kDa, and the increase in the intensity of fraction < 10 kDa (Figure 2C; Lanes 4-8). Finally, Corolase 7089 treatment decreased the intensity of bands with MM of 50, 55, 67 kDa while the appearance of lower MM bands at 25 and 28 kDa and under 6 kDa were observed after 1 h (Figure 2D; Lane 3). Longer digestion time had little impact on the SDS-PAGE profiles in which only the increased intensity in the fraction under 6 kDa was observed (Figure 2D; Lanes 4-8). Resistance of lentil and other legume proteins to proteases such as Protamex and Corolase 7089 has not been reported previously.

ACE inhibitory activity of lentil protein hydrolysates. ACE-inhibitory peptides are generally short amino acid sequences since the active site of ACE cannot accommodate large peptide molecules.[32] Therefore, samples were ultrafiltrated through 3 kDa cutoff membrane and permeate was used for ACE-inhibitory analysis. Non-hydrolyzed lentil protein concentrate showed a weak ACE-inhibitory activity (20% inhibition). ACE-inhibitory activity of hydrolyzates was significantly affected by the protease used and hydrolysis time (Table 1). Alcalase and Savinase hydrolyzates exhibited higher ACE inhibition (64-71% and 56-63%, respectively) than Protamex (31-46%) and Corolase 7089 (28-50%) digests, regardless of hydrolysis time. ACE-inhibitory efficacy is directly associated with chain length and peptide sequence. Short peptide chains containing between 3-12 amino acid residues and Tyr, Phe and Pro or hydrophobic amino acids are reported to be the most favorable sequences for ACE inhibition.[10] Alcalase and Savinase produced hydrolyzates with higher yield of shorter peptide sequences (Figure 2A and 2B) that joined to their specificity for aromatic or hydrophobic residues at position P1[22] is consistent with their higher ACE-inhibitory efficacy. ACE-inhibitory activity of hydrolyzates gradually increased

(P≤0.05) reaching maximum values after 1-3 h depending on the enzyme, while this activity decreased with extended hydrolysis time (P≤0.05) likely due to active amino acid sequences were cleaved by protease. IC_{50} values were calculated for A3 and S2 hydrolyzates, selected for their highest ACE-inhibitory activities. The selected hydrolysis time (3 h for Alcalase and 2 h for Savinase) was chosen because no significant improvements were found with further digestion times (P≥0.05). The A3 and S2 hydrolyzates exhibited significantly different (P≤0.05) IC_{50} values of 0.25±0.02 mg protein/mL and 0.18±0.02 mg protein/mL, respectively. It is worth noting the considerably higher ACE inhibitory activity of S2 hydrolyzates compared to A3 hydrolyzates (P≤0.05). These results suggest that Savinase should generate peptides with most favorable amino acid sequences than Alcalase. As far as we know, this is the first evidence showing the effectiveness of Savinase in releasing ACE-inhibitory peptides from legume proteins. IC_{50} values of ACE-inhibitory peptides in A3 and S2 hydrolyzates are higher when compared to activities of peptides released from lentil proteins by gastrointestinal enzymes (0.43-0.89 mg/mL).[14, 33, 34] Moreover, the ACE-inhibitory activity of S2 hydrolyzates found in this work is within the range of reported IC_{50} values for red and green lentil concentrates hydrolyzed by Alcalase/Flavourzyme, bromelain and papain (0.19-0.08 mg/mL), although it is noteworthy that much longer hydrolysis time (8 h) were used in the case of both plant proteases.[13] The ACE-inhibitory activity of A3 and S2 hydrolyzates are quite promising in comparison to activities reported for Alcalase hydrolyzates from different legumes such as cowpea, chickpea, mung bean and soybean exhibiting IC_{50} values ranging from 2.5 to 0.13 mg/mL.[23, 25, 27, 35]

Antioxidant activity of lentil protein hydrolysates. The effect of hydrolysis time and protease type on the potential antioxidant activity of lentil hydrolyzates was determined using the ORAC-FL method which reflects the peroxyl radical scavenging activity.[36] Antioxidant mechanism assayed with ORAC method is based on a hydrogen atom transfer mechanism. A radical initiator is used to generate

295 peroxyl radical ROO$^{\bullet}$ that abstracts a hydrogen atom from antioxidant molecules present in the sample.
296 Results in Table 2 indicate that ORAC values of non-hydrolyzed lentil proteins were significantly lower
297 compared to lentil hydrolyzates ($P \leq 0.05$). Hydrolysis time and protease used greatly affected the radical
298 scavenging activity of lentil protein hydrolyzates. Hydrolyzates produced using Alcalase, Savinase and
299 Corolase 7089 displayed a gradual increase in peroxyl scavenging activity reaching the highest values
300 after 4, 2 and 3 h, respectively ($P \leq 0.05$). In contrast, Protamex hydrolyzates showed the highest activity
301 ($P \leq 0.05$) at the shortest time (1 h), after which a gradual decrease was observed ($P \leq 0.05$). The
302 differences in antioxidant activity observed at different hydrolysis times could be attributable to the
303 different peptides and amino acid sequences released during hydrolysis. This leads to a difference in
304 their ability to act as donors of hydrogen atoms that may inhibit oxidative chain reactions or prevent
305 their initiation. Higher antioxidant activity potential of A4 and S2 hydrolyzates may be explained by the
306 higher efficiency of Alcalase and Savinase producing smaller peptides (Figures 2A and 2B). The
307 molecular weight of peptides is believed to play a key role in scavenging oxygen radicals. Recent
308 studies pointed out that cowpea and bean protein hydrolyzates radical scavenging activity increased with
309 decreasing molecular weight.[27, 37] In fact, identified antioxidant peptides derived from food proteins are
310 composed of 2-16 amino acid residues.[38] The higher efficiency of subtilases in releasing peroxyl
311 scavenging peptides observed in the present study is consistent with their preferential specificity to
312 cleave hydrophobic amino acids which have been shown to act as proton donors.[39] Comparison of the
313 ORAC values showed that S2 hydrolyzate exhibited the highest peroxyl scavenging activity (0.8 μmol
314 TE/mg protein) followed by A4 hydrolyzate (0.7 μmol TE/mg protein). These results indicate that
315 Savinase release antioxidant peptides with more favorable amino acid sequences than Alcalase. The
316 ORAC values of permeates < 3 kDa from S2 and A4 hydrolyzates was also analyzed. Radical
317 scavenging activity was 2-fold higher in these fractions compared to whole hydrolyzates. A4 permeates

318 exhibited 0.91±0.05 μmol TE/mg protein and S2 permeates exhibited 1.22±0.06 μmol TE/mg protein
319 suggesting that short peptides may be responsible for the potential antioxidant activity of protein
320 hydrolyzates in agreement with previous studies.[27, 37] To our knowledge, this is the first study on the
321 production of hydrolyzates with antioxidant activity from lentil proteins. The antioxidant activity
322 measured by ORAC-FL method of A4 and S2 hydrolyzates was more than two times higher compared
323 to Alcalase hydrolyzates from different soybean cultivars.[40] In addition, lentil hydrolyzates produced by
324 subtilases in the present study exhibited ORAC values within the range of reported values for common
325 vegetables (green pepper, spinach, purple onion, broccoli, beet and cauliflower) considered as leading
326 sources of antioxidant activity against peroxyl radicals (0.023-0.3 μmol TE/mg d.w.).[36]

327 **Peptide mass fingerprint of selected lentil hydrolyzates.** A4 and S2 hydrolyzates were selected
328 for MALDI-TOF analysis based on their highest biological activities. Figure 3 shows the mass spectra of
329 the 3 kDa permeate of A4 and S2 hydrolyzates. Comparison of the spectra showed similarities between
330 A4 and S2 hydrolyzates such as the presence of high intensity signals (m/z 1251.1-1255.5, m/z 1325.5-
331 1327.5, m/z 1340.5-1342.5, m/z 1652.8-1657.8 and m/z 2004.0-2007.1) referred to either matrix related
332 clusters or peptides that could represent concomitants including non-specific digestion products.[41, 42] It is
333 worth noting that A4 and S2 hydrolyzates showed differential peptide masses with reduced intensity (see
334 the inset mass peptide list in Figure 3). The different peptide profile observed for A4 and S2
335 hydrolyzates indicates that Alcalase and Savinase have different enzyme specificity which is consistent
336 with the above mentioned results.

337 **Identification of bioactive multifunctional peptides.** S2 hydrolysate was selected for peptide
338 identification based on its higher ACE-inhibitory and antioxidant activities and the 3kDa permeate was
339 analyzed by RP-HPLC-MS/MS. Table 3 shows the list of peptides identified containing in part of their
340 structure amino acid sequences with reported ACE-inhibitory (bolded sequences) and antioxidant

activity (underlined sequences) according to BIOPEP database. Identified peptides were fragments derived from lentil storage proteins (convicilin, vicilin, legumin and albumin), allergen Len c 1 and lectin having at the C-terminus residues such as alanine (A), valine (V), leucine (L), tyrosine (Y), phenylalanine (F) and tryptophan (W). The presence of some of these amino acids in ultimate position fulfils the rule proposed by Cheung et al.[43] about residues being preferred for ACE inhibitors and substrates. Moreover, peptides identified were characterized by the presence of several hydrophobic amino acids such as Ala (A), Pro (P). Val (V), Ile (I) Leu (L), Phe (F) Trp (W), Tyr (Y) and Met (M) which may act as proton donors .[39] Specifically, Tyr (Y) and Trp (W) have been reported as the main responsible for the antioxidant activity of peptides in the ORAC-FL model.[42, 45] In some peptides His (H) was also found which has been commonly associated with antioxidant activity due to its hydrogen-donating and radical-trapping imidazol ring.[39] Taking all together, the identification of the amino acid sequence of peptides in Savinase lentil hydrolysates have demonstrated their multifunctional properties that could make an important contribution in dietary interventions for prevention/therapy of hypertension. This is the first study showing an exhaustive characterization of the peptide fraction of lentil protein hydrolysates. So far, only three ACE-inhibitory peptides (KLRT, TLHGMV and VNRLM) released by sequential pepsin-pancreatin hydrolysis from lentil proteins were identified[33] and none of them were identified in the present work.

Stability of ACE-inhibitory and antioxidant peptides in selected lentil hydrolyzate to gastrointestinal digestion. There are numerous *in vivo* studies in animal models of hypertension and human clinical studies in hypertensive patients showing that oral administration of bioactive peptides from food proteins with ACE-inhibitory activity or foods containing them outcomes in the reduction of hypertension.[46] This body of evidence demonstrates that ACE-inhibitory peptides can resist gastrointestinal digestion to be absorbed in the intestine and ultimately reach the target molecule.

Nevertheless, gastrointestinal enzymes may cause structural degradation of bioactive peptides and loss of their bioactivity. Therefore, it is important to determine their stability to gastrointestinal digestion. For this purpose, the most potent peptide fraction (3 kDa permeate of S2 hydrolyzate) were further sequentially digested with pepsin and pancreatin to simulate gastrointestinal digestion. Some studies have reported a relation between peptide structure and bioavailability, thus, small hydrophobic peptides tend to be resistant to gastrointestinal digestion and, therefore, are generally absorbed.[47] Accordingly, based on the cleavage mechanism of Savinase, lentil hydrolyzates produced in the present study were hydrophobic and with molecular masses below 3 kDa, which support the potential bioavailability of such hydrolyzates. Figure 4 shows ACE-inhibitory and antioxidant activities of 3 kDa permeate of S2 hydrolyzate before and after *in vitro* gastrointestinal digestion. When peptides were subjected to *in vitro* gastrointestinal digestion IC_{50} was slightly lower (0.14 mg protein/mL) compared to that before digestion which indicates a higher ACE-inhibitory potency (0.18 mg protein/mL) ($P \leq 0.05$). In addition, antioxidant activity of peptides in the 3 kDa fraction increased 3 times after gastrointestinal digestion ($P \leq 0.05$). These results suggest that peptides in S2 hydrolyzates were either resistant or liberated new fragments with ACE-inhibitory and antioxidant activity after simulated gastrointestinal digestion. As consequence, peptides in S2 hydrolysate could preserve or improve its multifunctionality in the gastrointestinal tract.

In conclusion, this study reveals that subtilisins are the most suitable enzymes for lentil proteolysis and production of hydrolysates with ACE-inhibitory and antioxidant activities. The highest ACE-inhibitory and antioxidant activities were found in lentil hydrolysates treated with Savinase for 2 h at 40 ºC. Therefore, Savinase is the best choice for a cost-effective production of lentil multifunctional hydrolysates. Several peptides with amino acid sequences rich in hydrophobic amino acids were identified in these hydrolysates which make them potential contributors to the dual bioactivity detected.

Multifunctional hydrolyzates could encourage value-added utilization of lentil proteins as functional ingredients of nutraceuticals for prevention of hypertension and cardiovascular diseases.

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Notes

The authors declare no conflict of interest.

ABBREVIATIONS LIST

ACE: angiotensin I-converting enzyme; ACN: acetonitrile; DH: degree of hydrolysis; HPLC: high performance liquid chromatography with photodiode array detection; LTQ: linear trap quadrupole; MALDITOF: matrix-assisted laser desorption/ionization time of flight; MM: Molecular mass; ORAC: oxygen radical absorbance capacity; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; TE: Trolox equivalents; TFA: trifluoroacetic acid

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FIGURE CAPTIONS

Figure 1. Degree of hydrolysis (DH) of lentil proteins by different proteases and hydrolysis times. The experiments were performed in duplicate. Bars indicate the standard deviation A: Alcalase; S: Savinase; P: Protamex; C: Corolase 7089.

Figure 2. Sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of lentil hydrolyzates obtained by different proteases and hydrolysis times. Panel A, Alcalase; Panel B, Savinase; Panel C, Protamex; and Panel D, Corolase 7089 hydrolyzates. Lane 1: molecular weight marker Mark 12^{TM}; Lanes 2-8: 0, 1, 2, 3, 4, 5, and 6 h of hydrolysis, respectively.

Figure 3. Peptide mass fingerprint of 3 kDa permeates of lentil hydrolyzates. Panel A, A4 hydrolyzate; Panel B, S2 hydrolyzate. The mass spectra were obtained by MALDITOF in the reflector mode for positive ions. The respective table insets show differential peptide masses observed for each hydrolyzate.

Figure 4. Stability to gastrointestinal digestion of ACE inhibitory and antioxidant activity of 3 kDa permeates of A3 and S2 hydrolyzates. Lines represent ACE-inhibitory activity and columns show

547 antioxidant activity before and after *in vitro* gastrointestinal digestion. * indicate statistical difference at
548 $P < 0.05$; ** indicate statistical difference at $P < 0.001$ (Duncan test)

Table 1. Effect of hydrolysis time and protease type on ACE-inhibitory activity of lentil hydrolysates

	ACE Inhibition (%)[1]			
Time (h)	A	S	P	C
0	$20.34\pm1.48^{A}_{a}$	$20.49\pm0.53^{A}_{a}$	$21.20\pm4.02^{A}_{a}$	$20.17\pm0.1^{A}_{a}$
1	$64.37\pm1.57^{B}_{c}$	$56.73\pm1.54^{B}_{b}$	$46.52\pm3.84^{D}_{a}$	$41.51\pm0.12^{D}_{a}$
2	$64.37\pm0.25^{B}_{c}$	$63.35\pm2.41^{C}_{c}$	$41.04\pm1.17^{CD}_{a}$	$50.57\pm5.45^{E}_{b}$
3	$71.65\pm0.74^{D}_{c}$	$59.08\pm0.42^{B}_{b}$	$38.10\pm1.90^{C}_{a}$	$36.79\pm2.28^{CD}_{a}$
4	$70.37\pm2.93^{D}_{c}$	$59.08\pm1.61^{B}_{b}$	$40.50\pm1.42^{C}_{a}$	$36.23\pm4.42^{CD}_{a}$
5	$68.00\pm2.36B^{CD}_{c}$	$57.41\pm2.08^{B}_{b}$	$31.86\pm1.11^{B}_{a}$	$33.19\pm3.46^{BC}_{a}$
6	$67.74\pm1.98^{BC}_{c}$	$56.33\pm2.23^{B}_{b}$	$31.23\pm0.04^{B}_{a}$	$28.37\pm1.19^{B}_{a}$

Data indicate mean value ± standard deviation of two independent experiments. Different uppercase letters within column are significantly different ($P<0.05$, Duncan´s test). Different lowercase letters within row are significantly different ($P<0.05$, Duncan´s test).

A: Alcalase, S: Savinase, P: Protamex, C: Corolase 7089.

Protein concentration of samples in the ACE inhibition assay was 0.5 mg/mL.

Table 2. Effect of hydrolysis time and protease type on antioxidant activity of lentil hydrolysates

Time (h)	ORAC (µmol Trolox/mg protein)			
	A	S	P	C
0	$0.43\pm0.02^{A}{}_{a}$	$0.43\pm0.02^{A}{}_{a}$	$0.44\pm0.02^{A}{}_{a}$	$0.44\pm0.03^{A}{}_{a}$
1	$0.57\pm0.03^{B}{}_{bc}$	$0.52\pm0.03^{BC}{}_{a}$	$0.58\pm0.03^{E}{}_{c}$	$0.53\pm0.04^{BC}{}_{ab}$
2	$0.64\pm0.2^{C}{}_{c}$	$0.81\pm0.02^{F}{}_{d}$	$0.56\pm0.03^{CD}{}_{b}$	$0.50\pm0.04^{B}{}_{a}$
3	$0.62\pm0.03^{C}{}_{b}$	$0.75\pm0.07^{E}{}_{c}$	$0.55\pm0.04^{BCD}{}_{a}$	$0.54\pm0.04^{CD}{}_{a}$
4	$0.70\pm0.05^{D}{}_{c}$	$0.62\pm0.05^{D}{}_{b}$	$0.49\pm0.02^{B}{}_{a}$	$0.59\pm0.02^{D}{}_{b}$
5	$0.70\pm0.04^{D}{}_{b}$	$0.56\pm0.05^{C}{}_{a}$	$0.53\pm0.03^{BC}{}_{a}$	$0.55\pm0.03^{CD}{}_{a}$
6	$0.57\pm0.03^{B}{}_{c}$	$0.49\pm0.05^{B}{}_{a}$	$0.51\pm0.04^{AB}{}_{ab}$	$0.53\pm0.02^{BC}{}_{bc}$

Data indicate mean value ± standard deviation of two independent experiments. Different uppercase letters within column are significantly different ($P<0.05$, Duncan´s test). Different lowercase letters within row are significantly different ($P<0.05$, Duncan´s test).

A: Alcalase, S: Savinase, P: Protamex, C: Corolase 7089.

Table 3. Amino acid sequences found in 3 kDa permeates of S2 lentil hydrolysates

MH^+ (Da)	Peptide sequence	Protein	Accession
1252.78	**DLP**V**L**<u>**RW**</u>L	Legumin A	388256;483449;126161
1252.78	**DLP**V**L**<u>**RWL**</u>**K**<u>**L**</u>	Legumin A	388256;483449;126161
1927.07	DRRQEINKENVI<u>**V**</u><u>**K**</u>VS	Allergen Len c 1.0102	Q84UI0
835.47	EGGL<u>**LLP**</u>H	Convicilin	Q9M3X8
1982.11	EGSL<u>**LLPHY**</u>NSRAIVIVT	Vicilin	137582;1297072
1876.87	FEGT**V**<u>**F**</u>ENGIDA<u>**AY**</u>RST	Albumin-2	113570
977.57	INDK<u>YVL</u>L	Albumin-2	P86782
1237.72	**LF**INDK<u>YVL</u>L	Albumin-2	P86782
1002.55	NEDVI<u>**V**</u><u>**K**</u>VS	Allergen Len c 1.0101	Q84UI1
1831.99	NLERGDT<u>IKL</u>PAGT<u>**I**</u><u>**A**</u><u>**Y**</u>	Allergen Len c 1.0101	Q84UI1;Q84UI0
1945.07	NLERGDT<u>IKL</u>PAGT<u>**I**</u><u>**AY**</u>**L**	Allergen Len c 1.0101	Q84UI1;Q84UI0
2245.15	N**RF**QT<u>LY</u>ENENG**HIRL**LQ	allergen Len c 1.0101	42414629;29539109;29539111
1499.84	QEINKENVI<u>**V**</u><u>**K**</u>VS	Allergen Len c 1.0102	Q84UI0
2131.10	**RF**QT<u>**LY**</u>ENENG**HIRL**LQ	Allergen Len c 1.0101	42414629;29539109;29539111
1269.69	RLSAEY**VRL**<u>**Y**</u>	Legumin type B	126164;126166;126170
1725.01	RRQEINKENVI<u>**V**</u><u>**K**</u>V	Allergen Len c 1.0102	Q84UI0
1927.07	RRQEINKENVI<u>**V**</u><u>**K**</u>VS	Allergen Len c 1.0102	Q84UI0
1098.63	SL**DLP**V**L**<u>**RW**</u>	legumin A	483449;126161
1212.72	SL**DLP**V**L**<u>**RW**</u>L	legumin A	483449;126162
1421.80	SVEIKEGSL<u>**LLP**</u>H	Vicilin	137582;1297072
1698.91	SVEIKEGSL<u>**LLPHY**</u>N	Vicilin	137582;1297072
1785.94	SVEIKEGSL<u>**LLPHY**</u>NS	Vicilin	137582;1297072
1377.74	SVEINEGGL<u>**LLP**</u>H	Convicilin	7688242
1540.80	SVEINEGGL<u>**LLPHY**</u>	Convicilin	Q9M3X8
1897.98	SVEINEGGL<u>**LLPHY**</u>NSR	Convicilin	Q9M3X8
1817.93	<u>SWN</u>LQNGERANVV**IA**F	Lectin	Q93WH6;Q93X49
1312.76	TSL**DLP**V**L**<u>**RW**</u>L	legumin A	483449;126161
1512.88	TVTSL**DLP**V**L**<u>**RW**</u>L	legumin A	483449;126161
2538.43	VEIKEGSL<u>**LLPHY**</u>NSR	Vicilin	137582;1297072
1290.70	VEINEGGL<u>**LLP**</u>H	Convicilin	7688242
1634.91	VNSVEIKEGSL<u>**LLP**</u>H	Vicilin	137582;1297072
2155.15	VNSVEIKEGSL<u>**LLPHY**</u>NSR	Vicilin	137582;1297072

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) and Wu et al.[48]

Figure 1

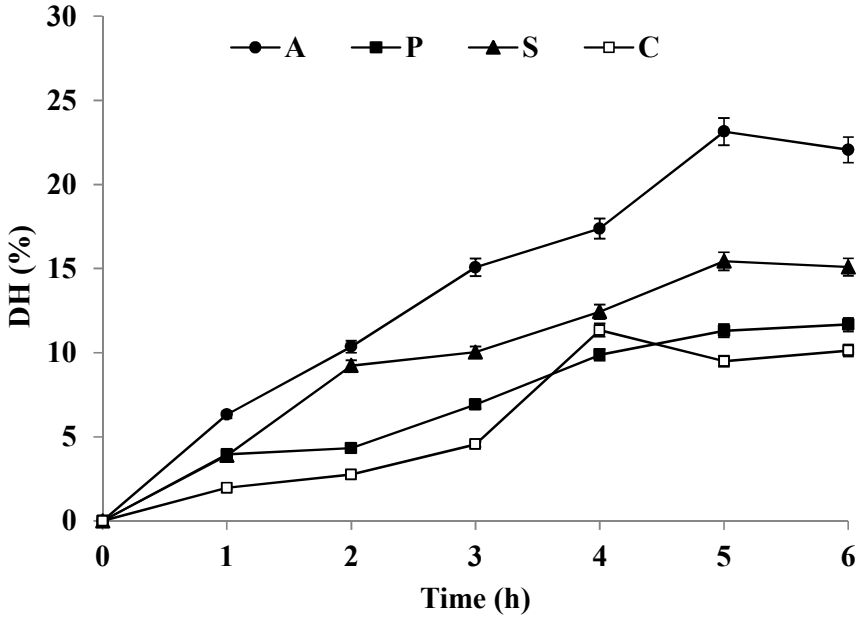
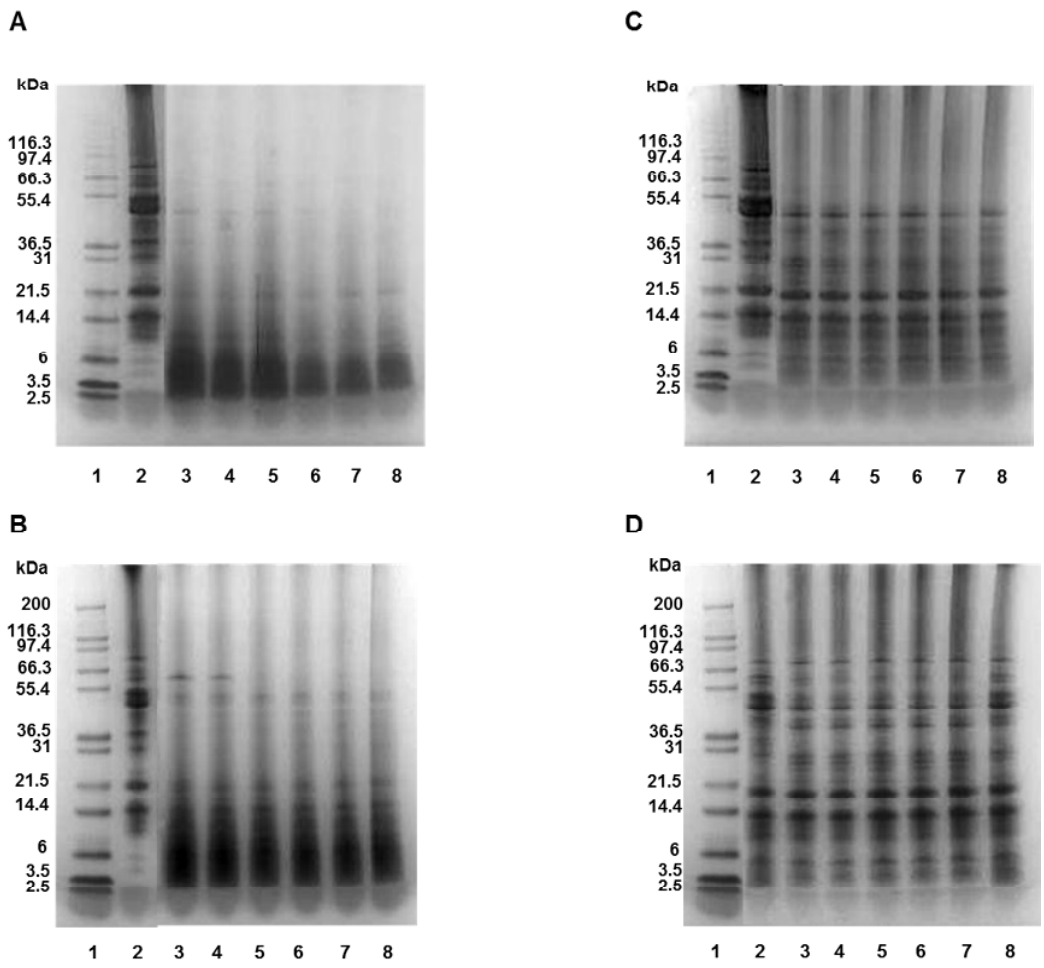


Figure 2


A
kDa
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
6
3.5
2.5
1 2 3 4 5 6 7 8
C
kDa
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
6
3.5
2.5
1 2 3 4 5 6 7 8
B
kDa
200
116.3
97.4
66.3
55.4
36.5
31
21.5
14.4
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3.5
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1 2 3 4 5 6 7 8
D
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200
116.3
97.4
66.3
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31
21.5
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3.5
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1 2 3 4 5 6 7 8

Figure 3.

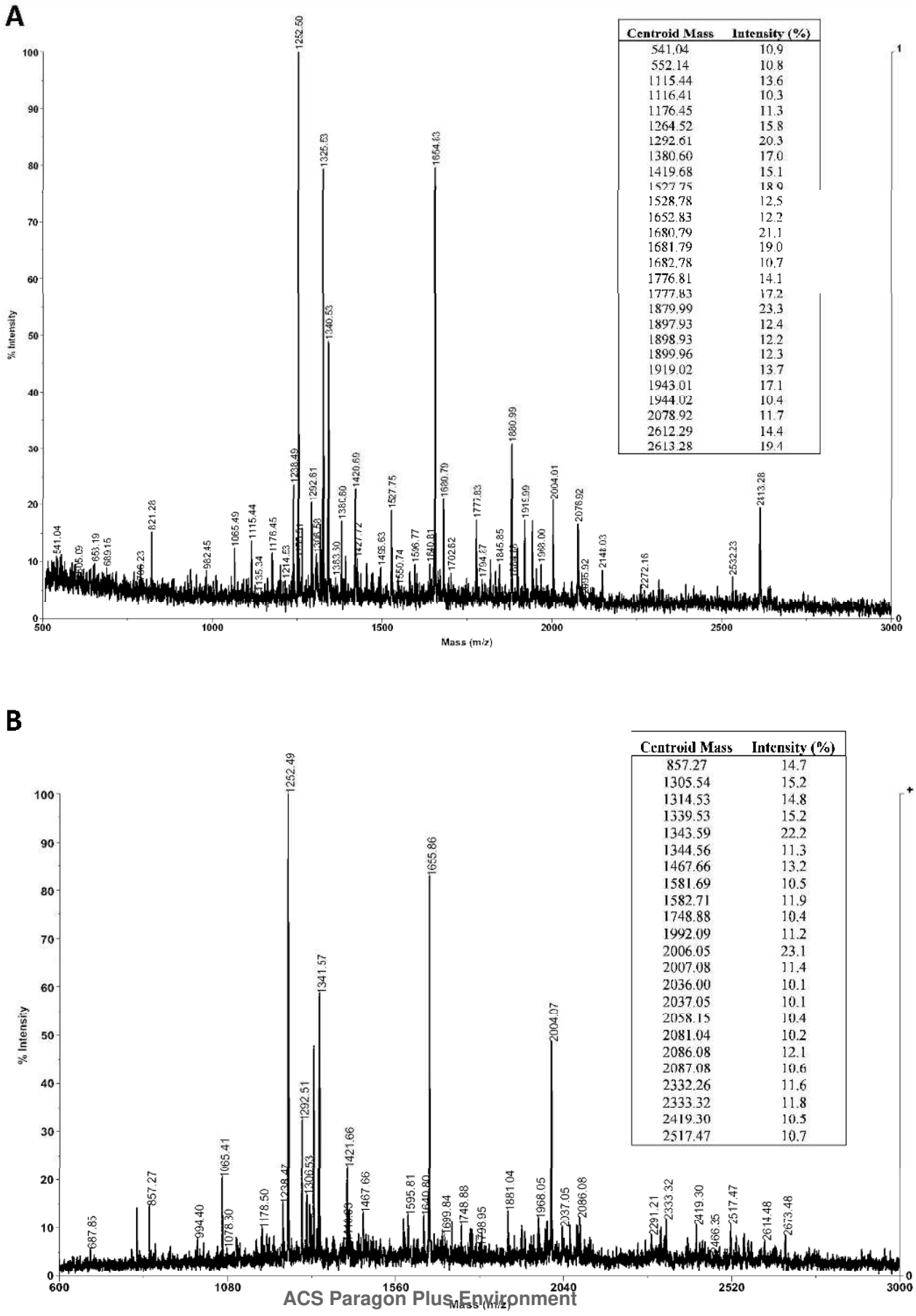
A
Centroid Mass Intensity (%)
541.04 10.9
552.14 10.8
1115.44 13.6
1116.41 10.3
1176.45 11.3
1264.52 15.8
1292.61 20.3
1380.60 17.0
1419.68 15.1
1527.75 18.9
1528.78 12.5
1652.83 12.2
1680.79 21.1
1681.79 19.0
1682.78 10.7
1776.81 14.1
1777.83 17.2
1879.99 23.3
1897.93 12.4
1898.93 12.2
1899.96 12.3
1919.02 13.7
1943.01 17.1
1944.02 10.4
2078.92 11.7
2612.29 14.4
2613.28 19.4
% Intensity
Mass (m/z)
B
Centroid Mass Intensity (%)
857.27 14.7
1305.54 15.2
1314.53 14.8
1339.53 15.2
1343.59 22.2
1344.56 11.3
1467.66 13.2
1581.69 10.5
1582.71 11.9
1748.88 10.4
1992.09 11.2
2006.05 23.1
2007.08 11.4
2036.00 10.1
2037.05 10.1
2058.15 10.4
2081.04 10.2
2086.08 12.1
2087.08 10.6
2332.26 11.6
2333.32 11.8
2419.30 10.5
2517.47 10.7
% Intensity
Mass (m/z)

Figure 4.

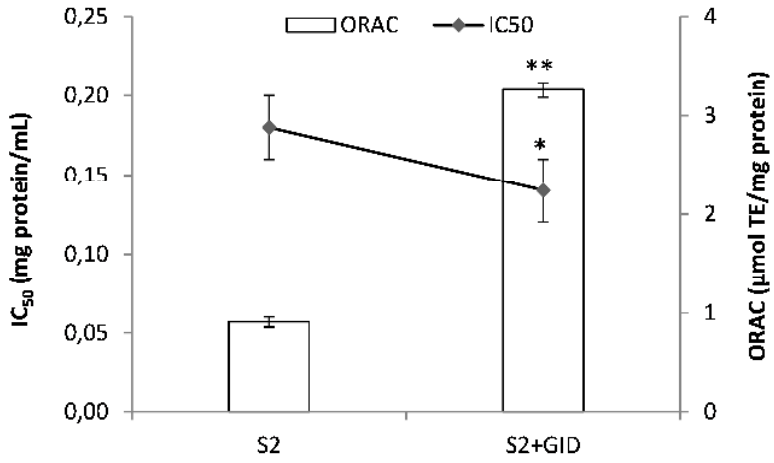
ORAC
IC50
**
*
0,25
0,20
0,15
0,10
0,05
0,00
4
3
2
1
0
IC50 (mg protein/mL)
ORAC (µmol TE/mg protein)
S2
S2+GID

TABLE OF CONTENT GRAPHIC