**Peptidomic analysis of antioxidant and ACE-inhibitory peptides obtained from
tomato waste proteins fermented using *Bacillus subtilis***

**Ali Moayedi¹, Leticia Mora^{*2}, Maria Concepción Aristoy², Mohammad Safari^{3,4},
Maryam Hashemi⁵, Fidel Toldrá²**

¹Department of Food Science and Technology, Gorgan University of Agricultural
Sciences and Natural Resources, Gorgan, Iran

²Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Avenue Agustín
Escardino, 46980, Paterna, Valencia, Spain

³Department of Food Science, Engineering and Technology, University of Tehran,
Karaj, Iran

⁴Center of Excellence for Application of Modern Technology for Producing Functional
Foods and Drinks, University of Tehran, P.O. Box 4111, 31587-77871 Karaj, Iran

⁵Department of Microbial Biotechnology and Biosafety, Agricultural Biotechnology
Research Institute of Iran (ABRII), AREEO, P.O. Box 3135933151, Karaj, Iran

*Corresponding author address: Leticia Mora (lemoso@iata.csic.es) Tel:
+34963900022 ext. 2114; Fax: +34963636301

Abstract

In this study, tomato seeds were obtained as tomato processing industry by-products and fermented with proteolytic *Bacillus subtilis*. The resulting peptide mixture was fractionated and purified through different chromatographic steps, and antioxidant and ACE-inhibitory activities of the obtained fractions were evaluated. Most active peaks were identified using nano-liquid chromatography coupled to mass spectrometry in tandem (nLC-MS/MS). From all the identified peptides, an important number was smaller than 1000 Da and showed different aromatic and hydrophobic amino acid residues. The sequences of identified peptides were novel but some of them showed active domains previously reported in bioactive peptides. The hexapeptide DGVVYY showed an IC₅₀ value of 2 µM in angiotensin-I converting enzyme (ACEI) inhibitory activity whereas the pentapeptide GQVPP proved a 97% of DPPH activity at 0.4 mM. The results revealed that *B. subtilis* fermentation of tomato by-products could be a good strategy to obtain added-value peptides that might be used as an ingredient in functional foods and nutraceuticals.

Keywords: Fermentation, Protein waste, Bioactive peptides, Purification, Peptide sequencing

1. Introduction

Bioactive peptides have been described to be present in many fermented and functional foods. These small protein fragments have 2-20 amino acid residues and exert their biological activity after being released from the parent proteins (Hartmann & Meisel, 2007; Udenigwe & Aluko, 2012). Depending on amino acid composition and sequence length, bioactive peptides could display different activities such as antihypertensive, antioxidant, immunomodulatory, opioid, anti-inflammatory or antimicrobial (Shahidi & Zhong, 2008).

During the last decade, many ACE-inhibitory and antioxidant peptides have been produced and characterised. Main trends have been focused on the use of agro-industrial waste or by-products as a source for the generation of bioactive peptides in order to maximise the economic return and contribute to the environmental sustainability. Regarding this, olive seeds (Esteve, Marina, & García, 2015), date seeds (Ambigaipalan, Al-Khalifa, & Shahidi, 2015), cherry seed (García, Endermann, González-García, & Marina, 2015), palm kernel cake (Chang, Ismail, Yanagita, Esa, & Baharuldin, 2015), and fish and marine processing waste (Bougatef et al., 2010) have been hydrolysed by using commercial enzymes to produce bioactive peptides.

Despite enzymatic hydrolysis with commercial enzymes like trypsin, pepsin, Alcalase, Flavourzyme and Thermolysin has been the most widely used method to digest parent proteins and generate peptides, the microbial fermentation by using proteolytic species has also resulted to be a successful strategy (Fakhfakh, Ktari, Siala, & Nasri, 2013; Jemil, Mora, et al., 2016; Niu, Jiang, & Pan, 2013; Yang et al., 2016). The simultaneous action of different proteases showing different specificities in the fermentation medium enhances the chance of small peptides generation. Among the microbial species,

different species of *Bacillus* genus may have a unique place due to their high proteolytic properties. In this respect, Jemil, Mora, *et al.* (2016) tested fermentation products of Sardinelle protein with *Bacillus subtilis* A26 and *Bacillus amyloliquefaciens* An6 to produce antioxidant and ACE-inhibitory peptides whereas Zhang, *et al.* (2014) used *B. subtilis* to hydrolyse peanut proteins. In another study, Jemil Abdelhedi, *et al.* (2016) prepared antibacterial peptides from zebra blenny muscle proteins after fermentation with *B. mojavensis* A21. Fakhfakh, *et al.* (2013) also revalorised wool waste by generating bioactive peptides using *B. pumilus* fermentation. In previous papers, the optimization of fermentation conditions (Moayedi, Hashemi, & Safari, 2015) and the effects of amino acid composition and molecular weight distribution on antioxidant and ACE-inhibitory activity of resulting peptides (Moayedi, Mora, Aristoy, Hashemi, Safari, & Toldrá, 2017) was described. In this study, the isolation and identification of most active peptide sequences obtained from the fermentation of tomato seeds with *B. subtilis* have been done using different chromatographic, mass spectrometry, and bioinformatic tools. The *in vitro* activities of synthesized peptide sequences have also been evaluated.

2. Materials and Methods

2.1. Chemicals and reagents

Abz-Gly-p-nitro-phe-pro-OH trifluoroacetate salt used as substrate was obtained from Bachem AG. (Bubendorf, Switzerland). 2, 2'-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT) and ACE enzyme (from rabbit lung) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Microbial culture media, reagents for ferric reducing assay, HPLC grade acetonitrile, hydrochloric acid 37%, and

trifluoroacetic acid were purchased from Scharlau (Barcelona, Spain) and Merck Company. Regarding mass spectrometry analysis, water, acetonitrile and formic acid were used of MS grade and obtained from Scharlau (Barcelona, Spain). All other chemicals were of analytical grade.

2.2. Fermentation and preparation of TSM hydrolysates

Tomato pulp containing skins and seeds was supplied by Golgashte Shirin plant (Ghazvin, Iran). After removing skins, the seeds were sun-dried, ground in a blender and defatted using Soxhlet method with n-hexane as solvent. The resulting powdered tomato seed meal (TSM) was used as the only source of carbon and nitrogen in a submerged fermentation with the proteolytic strain *B. subtilis* A14h (Moayedi et al., 2015). Fermentation was carried out in 250 mL flasks containing 5% of Trichoderma-selecting medium (TSM), K₂HPO₄ (0.05%, w/v), MgSO₄ (0.01%, w/v), and CaCO₃ (0.16% w/v), and were inoculated with 2% (v/v) of *B. subtilis* fresh culture (1×10^8 CFU/mL) and kept for 24 h at 37 °C under agitation conditions. After incubation, the fermented media was heated in boiling water for 15 min in order to stop the enzymatic reaction. Then, sample was centrifuged at 12000 rpm (Suprema 25, TOMY, Japan) for 10 min, and the supernatant was collected, lyophilised, and stored at -20 °C until use. This lyophilised powder will be referred as TSMH (tomato seed meal hydrolysate).

Four grams of lyophilised TSMH was dissolved in 20 mL of 0.01N HCl and homogenised in a Stomacher at 4 °C for 8 min. The homogenate was centrifuged, and the supernatant was filtered through glass wool. In order to precipitate the proteins, the filtered supernatant was mixed with 3 volumes of ethanol and kept in cold (4-5 °C) for 20 h. Then, the mixture was centrifuged (12000 g for 20 min at 4 °C), and the supernatant was lyophilised after removing the ethanol by using a rotary evaporator.

2.3. Fractionation and purification using size-exclusion chromatography

The lyophilised sample was dissolved in 15 mL of 0.01N HCl, filtered through 0.45 µm syringe filter and loaded (5 mL) onto a gel filtration column packed with Sephadex G-25 gel. The mobile phase was 0.01 N HCl at a flow rate of 15 mL/h and a total of 130 fractions of 5 mL were collected using an automatic collector (Escudero, Aristoy, Nishimura, Arihara, & Toldrá, 2012). In order to determine the peptide profile of the samples, the absorbance of fractions was measured at 214 and 280 nm. All the fractions showing absorbance at the mentioned wavelengths were evaluated for their antioxidant and ACE-inhibitory activities, and the fractions with the highest activities were lyophilised again and stored for the next purification step.

2.4. High performance liquid chromatography (RP-HPLC) fractionation in reversed-phase

The most active fractions obtained after SEC separation were further fractionated according to their relative hydrophobicity using a HPLC (Agilent 1100 HPLC system, Agilent Technologies, CA, USA) equipped with a reversed-phase Symmetry C18 column (250×4.6 mm, 5µm). RP-HPLC conditions were similar to that previously described by Escudero, Mora, Fraser, Aristoy, Arihara and Toldrá (2013) with minor changes. Lyophilised samples were resuspended in bi-distilled water (90 mg/mL), filtered through 0.22 µm membrane filter, and manually injected (300 µL) into the column. The mobile phases and separation conditions were: solvent A; 0.1% trifluoroacetic acid (TFA) and solvent B; 0.085% TFA in 60:40, v/v, acetonitrile:water, with linear gradient of 0 to 50% of solvent B in 54 min at a flow rate of 1 mL/min. The absorbance was monitored at 280 nm and 1 mL fractions were collected using an automatic collector. Fractions were individually tested for DPPH

scavenging, ferric reducing power and ACE-inhibitory activities and the most active fractions were chosen for peptide identification. A flow diagram showing the different steps of this study is schemed in Fig. 1.

2.5. Peptide identification by tandem mass spectrometry

Peptide identification was done using a nano-liquid chromatography system (Eksigent of AB Sciex, CA) coupled to a quadrupole-time-of-flight (Q-ToF) mass spectrometer (TripleTOF 5600+; AB Sciex Instruments, Framingham, MA, USA) equipped with a nano-electrospray ionisation source (nano-ESI). Peptide identification was done as previously published by Lassoued, *et al.* (2016).

The data analysis was done using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA, USA). It uses Mascot search engine and the interpretation of spectra was done under these parameters: ‘other Chordata’ taxonomy, NCBIInr database, no post translational modifications, and tolerance of 100 ppm in MS and 0.5 Da in MS/MS. All identified sequences were $P < 0.05$. BIOPEP database was used in the search of similar sequences previously identified showing ACE inhibitory activity (<http://www.uwm.edu.pl/biochemia/index.php/pl/biopep>).

2.6. Peptide synthesis

According to peptide length and amino acid composition, a total of 13 of the identified peptides were chosen and synthesized by Gene-script corporation company (Piscataway, NJ, USA). The synthesized peptides were evaluated in terms of their antioxidant and ACE-inhibitory activities.

2.7. Determination of ACE inhibitory activities

A fluorescence-based method was used to determine ACE-inhibitory activities of peptide fractions and pure sequences (Sentandreu & Toldrá, 2006). Briefly, 50 μ L of 3 mU/mL ACE in Tris-base buffer (150 mM, pH 8.3) was added to 50 μ L of sample, and

the mixture was pre-incubated at 37 °C for 10 min. The reaction was started by adding to the mixture 200 µL of 10 mM Abz-Gly-Phe-(NO₂)-Pro previously dissolved in 150 mM Tris–HCl buffer (pH 8.3) and containing 1.125 M NaCl. The fluorescence intensity was measured using excitation and emission wavelengths of 355 and 405 nm, respectively, during incubation for 60 min at 37 °C. Bidistilled water and Captopril (0.1mg/mL) were used as negative and positive control, respectively. ACE inhibitory activity was expressed as percentage and measurements were done in triplicate (n=3). IC₅₀ value was calculated as the concentration of peptide needed to obtain a 50% of ACE-inhibitory activity. ACE inhibitory activity was calculated using the following equation:

$$\%ACE \text{ inhibitory activity} = (1 - \frac{F_s}{F_c}) \times 100$$

Where F_s is the fluorescence intensity of inhibitory-contained sample and F_c the fluorescence intensity of negative control.

2.8. Determination of antioxidant activities

2.8.1. DPPH scavenging activity

The DPPH scavenging activity of peptide fractions was determined according to the protocol described by Jemil, et al. (2014) with minor modifications. Thus, 190 µL of sample, 220 µL of ethanol and 95 µL of DPPH solution in ethanol (2%, w/v) were mixed and incubated for 60 min in the dark. Then, the absorbance was measured at 517 nm. BHT was used as positive control and bidistilled water as negative control. The percentage of DPPH scavenging activity was calculated according to the following equation:

$$\%DPPH \text{ scavenging activity} = (1 - \frac{A_s}{A_c}) \times 100$$

Where, A_s is the absorbance of sample and A_c is the absorbance of the control.

2.8.2. Ferric reducing power capacity

The reducing power (on Fe (III)) was assayed spectrophotometrically as described by Moayedi, *et al.* (2017). Briefly, a mixture of peptide sample (100 μ L) with 250 μ L of potassium phosphate buffer (0.2M, pH 6.6), and 250 μ L of 1% (w/v) potassium ferricyanide was incubated for 30 min at 50°C. Then, 250 μ L of 10% trichloroacetic acid (TCA) was added, mixed, and centrifuged at 12000 rpm for 10 min. Then, 250 μ L of the supernatant were taken and 250 μ L of distilled water and 50 μ L of 0.1% (w/v) of ferrous chloride were added. The absorbance was measured immediately at 700 nm. A sample containing BHT was used as positive control.

3. Results and Discussion

3.1. Fractionation using size exclusion chromatography (SEC)

The peptides generated during fermentation were firstly separated using SEC on a Sephadex G-25 column as it is showed in Figure 2, and ACE-inhibitory and antioxidant activities were tested in all the obtained fractions. In Figure 2a, fractions 46-59 (elution volumes from 225 to 295 mL) showed ACE-inhibitory activities higher than 80%. On the other hand, fractions 47-57 (corresponding to elution volumes from 230 to 285 mL) showed DPPH scavenging activities higher than 50%, including fraction 50 that scavenged the 90% of DPPH (Figure 2b). Similar results were obtained in the ferric reducing power assay, as the highest absorbance at 700 nm was observed for fractions 48-58 (Figure 2c). Accordingly, fractions from 46 to 56 corresponding to elution volumes from 225 ml to 280 ml and potentially containing the most active peptides, were pooled together, lyophilised and kept in freezing conditions for further purification by RP-HPLC.

3.2. Reversed-phase high performance liquid chromatography separation

RP-HPLC is one of the most suitable methods used in the separation of peptides in protein hydrolysates able to provide very useful information about their relative hydrophobicity. The mixture of most active peptides obtained from SEC purification was injected into the RP-HPLC system. In the separation process, a total of 60 fractions were collected, lyophilised and used to evaluate the antioxidant and ACE-inhibitory activity.

Results of RP-HPLC separation and the activity of fractions are shown in Figure 3. Thus, the ACE-inhibitory activity reached the 80% in the fractions eluting from 4 to 5 and 15 to 16 min. Regarding DPPH scavenging test, several fractions showed activities up to 67% despite the highest activity was observed for the fractions eluted from 4 to 5, and 52 to 53 min. On the other hand, fractions eluted from 44 to 45, and 52 to 53 min showed the highest ferric reducing power. According to these results, fractions eluting from 4 to 5 min could contain multifunctional peptides showing both antioxidant and ACE-inhibitory activities. Multifunctional peptides have been identified in some previous studies (Cheung & Li-Chan, 2017; García-Mora et al., 2016). Since RP-HPLC separates peptides based on their relative hydrophobicity, peptides showing longer retention times are expected to contain more hydrophobic or aromatic amino acids in their structure. The DPPH scavenging activity and ferric reducing power determined in fractions 44, 45, 52 and 53 may be related to the presence of aromatic amino acids in peptide sequences of these fractions. Similar results were reported by Pownall, Udenigwe, and Aluko (2010) which observed that later eluted fractions in RP-HPLC contained peptides with the hydrophobic amino acids Leu, Phe, Val and Trp, and displayed the highest radical scavenging activity and metal binding capacity.

Based on these results, the most active fractions from RP-HPLC purification with retention times 4, 5, 15, 16, 44, 45, 52 and 53 min were prepared for peptide sequencing and identification with nLC-MS/MS. Thus, each 2 consecutive fractions were placed in one new fraction: F1 (fractions eluted from 4 to 5 min), F2 (fractions eluted from 15 to 16 min), F3 (fractions eluted from 44 to 45 min) and F4 (fractions eluted from 52 to 53 min).

3.3. Identification of active peptides by tandem mass spectrometry

Molecular mass and amino acid sequence of peptides in fractions F1, F2, F3 and F4 were determined using nLC-MS/MS as it is shown in Table 1. The molecular masses of the identified peptides ranged between 400 to 3600 Da. In this respect, a 27% of the identified peptides were below 600 Da with 6 or less amino acids in length which agrees with previously published results that describe most bioactive peptides to be between 3 and 20 amino acid residues (Korhonen & Pihlanto, 2003). The size of identified peptides in this study would enhance their chance to cross intact the intestinal barrier, reach the blood stream, and exert their biological activity in target points of the organism (Mora, Escudero, Fraser, Aristoy, & Toldrá, 2014). In addition, most of the identified peptides contain aromatic and hydrophobic amino acid residues at their C-terminal. This fact may be attributed to the action of *B. subtilis* alkaline serine proteases (mainly subtilisin) that show preference for aromatic or hydrophobic residues at position P1 of cleavage site (García-mora et al., 2016; Moayedi et al., 2016).

The sequences of identified peptides reveal the contribution of different proteolytic enzymes produced by *B. subtilis* A14 during fermentation. As an example, peptides GQVPP, GQVPPQ and IGQVPP evidence the action of carboxypeptidases and aminopeptidases. Also, the loss of VH from VLYQFVH to obtain the peptide VLYQF

may be attributed to the action of dipeptidyl peptidases. Moreover, the endopeptidase activity of some of the proteolytic enzymes secreted by *B. subtilis* was also confirmed with the simultaneous identification of the peptides AATPKP and AAPVK, as a result of AATPKPAAPVK fractionation.

On the other hand, some of the identified peptides were found to share active domains with previously reported antioxidant and ACE-inhibitory peptides according to BIOPEP database (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008), as it is highlighted in Table 1. As an example, VPP, a well-known tripeptide for its ACE-inhibitory activity *in vitro* and antihypertensive effects *in vivo* and isolated from bovine beta casein (Nakamura, Yamamoto, Sakai, Okubo, Yamazaki, & Takano, 1995) have been detected as a part of the sequence of some of the identified peptides (VPPATQ, VPPKG, VPPAT, DVPPYM, and DVPPY). On the other hand, peptide sequences AAIGPHH, PAIGPHHLG, PHHIA, VPHHI and VALGVPHH identified in fractions F1, F3 and F4, contained PHH domain, which has shown to be antioxidant (Minkiewicz et al., 2008). This active domain has been observed in some peptide sequences from soy protein and tuna (Minkiewicz et al., 2008). The tripeptide YFY (Minkiewicz et al., 2008) has also been reported as part of the sequence in several peptides showing antioxidant or ACE-inhibitory activity. For example, AYFYYP, AYFYPE, and LAYFYYP were obtained from casein and described to exert ACE-inhibitory activity (Yamamoto, Akino, & Takano, 1994), whereas YFYYPQL was defined as antioxidant peptide (Minkiewicz et al., 2008). In this study, fractions F2 and F4 contained peptides showing YFY in their structure. Moreover, PLP has been found in human beta casein and reported in several bioactive peptide sequences showing ACE-inhibitory activity (Minkiewicz et al., 2008). In the current study, the domain PLP was found in peptides HPLPP, GKPLP, EPPLPP and EPPLP.

Regarding ACE-inhibitory activity, IC₅₀ values were calculated for all synthesized peptides. The highest ACE-inhibitory activity was found to be for peptide DGVVYY that resulted in an IC₅₀ value of 2 µM. In this respect, the ACE-inhibitory activity of DGVVYY peptide has resulted higher in comparison with previously reported activities of bioactive peptides generated from food hydrolysates. As an example, IC₅₀ for WPERPPQIP (from walnut protein hydrolysate), VVLYK (from palm kernel glutelin-2 hydrolysate), AYFYFP, LAYFYP and AYFYP, all from casein sub-fractions, were reported to be 25 µg/mL (M. Liu et al., 2013), 553.9 µM (Zheng, Li, Zhang, Ruan, & Zhang, 2017), 106, 65 and 1001 µM (Yamamoto et al., 1994), respectively.

The ACE-inhibitory activity is highly influenced by the presence of hydrophobic and branched-chain amino acids in the peptide structure (Girgih, He, Malomo, Offengenden, Wu, & Aluko, 2014; Hernández-Ledesma, del Mar Contreras, & Recio, 2011; Lassoued et al., 2015). It has been suggested that bulky aromatic amino acids increase hydrophobic interactions with enzymes (Girgih et al., 2014). The amino acids located in the three positions close to C-terminal is also a determining factor for competitive binding to ACE active site and therefore to exert inhibitory activity (Hernández-Ledesma et al., 2011; Lassoued, Mora, Nasri, Jridi, et al., 2015; Shahidi & Zhong, 2008). Therefore, the ACE inhibitory activity of DGVVYY could be related to the presence of Val, a branched amino acid with high affinity for ACE active sub-site (Girgih et al., 2014), and Tyr, an aromatic residue.

Furthermore, other powerful ACE-inhibitory peptides were PGVPWP and VPPLYPN with IC₅₀ of 19 and 21 µM, respectively. Both peptides show Pro in their sequence and VPP tripeptide has been previously reported as an effective ACE inhibitor (Nakamura et al., 1995). The hexapeptides PGLPKK and GYFPPP also showed inhibitory activities against ACE with an IC₅₀ value of 32 µM. Some studies have pointed out the promoting

role of positively-charged and basic amino acids (Lys and Arg) at C-terminal on ACE-inhibitory activity of peptides (Shahidi & Zhong, 2008; Zheng et al., 2017). This situation also occurs in PGLPKK peptide showing two Lys residues at C-terminal position. Finally, peptide VALGVPHH also showed a low IC₅₀ value (33μM).

The DPPH radical-scavenging activity and ferric-reducing power of synthesized peptides were determined and the results are shown in Table 2 and Figure 4. In this respect, the peptide GQVPP showed the highest DPPH radical-scavenging activity that resulted in 97% at 0.4 mM. The peptide GQVPP also showed the highest ferric reducing power with an absorbance value of 0.95 at 0.5 mM.

Antioxidant peptides identified in protein hydrolysates from cherry seed (García et al., 2015), sardinelle (Jemil, Mora, et al., 2016), oil palm kernel (Chang et al., 2015), and hemp seed (Girgih et al., 2014) were described to show in their sequences aromatic and hydrophobic amino acids residues as well as resulted smaller than 10 amino acids length. The antioxidant activity of GQVPP peptide (97% at 0.4 mM) was higher than NVPVYEGY (IC₅₀= 1.41 mM) and AGRDLTDY (1.54 mM), which have been recently identified in *Bacillus*-fermented sardinelle protein (Jemil, Mora, et al., 2016), and higher than that described for peptides WVYY and PSLPA, both from hemp seed protein hydrolysate with 67% and 58% of activity at 0.5 mg/mL, respectively, (Girgih et al., 2014). However, its activity is approximately comparable with LYEGY (0.217 mM), an antioxidant peptide from *Macraa veneriformis* protein hydrolysate (Liu et al., 2015).

In addition to the effective role of aromatic and hydrophobic amino acids (Girgih et al., 2014) in antioxidant activity, acidic amino acids and their amide forms (Glu, Gln, Asp and Asn) have been reported to show a positive contribution in antioxidant activity of

peptides (Ghribi et al., 2015; Girgih et al., 2014; Udenigwe & Aluko, 2012). Therefore, the DPPH radical scavenging activity and ferric reducing power of GQVPP peptide could be related to the presence of Gln, Val and Pro.

4. Conclusion

In the current study, *B. subtilis* fermentation was used to generate bioactive peptides that were encrypted in proteins from tomato seeds. The resulting peptide mixture was fractionated according to their size and relative hydrophobicity. After each purification step, ACE-inhibitory and antioxidant activity was measured. Peptide identification with mass spectrometry in tandem revealed that many of the peptides contained in the most active fractions were below 600 Da, with 5-6 amino acid residues in length. There were six peptides with IC_{50} below $33\mu M$, being DGVVYY ($IC_{50} = 2\mu M$) the most promising peptide for inhibiting ACE activity. On the other hand, GQVPP showed excellent antioxidant activity (97% DPPH scavenging activity at 0.4 mM). The results revealed that proteolytic enzymes from *B. subtilis* effectively cleaved parent proteins of tomato seeds into short peptides showing good antioxidant and ACE inhibitory activities. However, although amino acids compositions and their sequence in the peptide structure greatly influences on peptide activity, it does not guarantee the expected bioactivity.

358

359

360

361

362

363 **5. Acknowledgement**

364 This work was funded by Grant of Ministry of Science, Research and Technology of
365 Iran and Emerging Research Group Grant from Generalitat Valenciana in Spain
366 (GV/2015/138). Also a Juan de la Cierva de Incorporación postdoctoral contract to Dr.
367 Mora is acknowledged. Q-ToF nLC-MS/MS was carried out in the SCSIE University of
368 Valencia Proteomics Unit (Spain), a member of ISCIII ProteoRed Proteomics Platform.
369 Authors would like to thank Carolina Diaz Noriega for her beneficial helps.

370

371

372

373

374

375

References

- Ambigaipalan, P., Al-Khalifa, A. S., & Shahidi, F. (2015). Antioxidant and angiotensin I converting enzyme (ACE) inhibitory activities of date seed protein hydrolysates prepared using Alcalase, Flavourzyme and Thermolysin. *Journal of Functional Foods*.
- Bougatef, A., Nedjar-Arroume, N., Manni, L., Ravallec, R., Barkia, A., Guillochon, D., & Nasri, M. (2010). Purification and identification of novel antioxidant peptides from enzymatic hydrolysates of sardinelle (*Sardinella aurita*) by-products proteins. *Food Chemistry*, 118(3), 559–565.
- Chang, S. K., Ismail, A., Yanagita, T., Esa, N. M., & Baharuldin, M. T. H. (2015). Antioxidant peptides purified and identified from the oil palm (*Elaeis guineensis* Jacq.) kernel protein hydrolysate. *Journal of Functional Foods*, 14, 63–75.
- Cheung, I. W. Y., & Li-Chan, E. C. Y. (2017). Enzymatic production of protein hydrolysates from steelhead (*Oncorhynchus mykiss*) skin gelatin as inhibitors of dipeptidyl-peptidase IV and angiotensin-I converting enzyme. *Journal of Functional Foods*, 28, 254–264. <http://doi.org/10.1016/j.jff.2016.10.030>
- Escudero, E., Aristoy, M.-C., Nishimura, H., Arihara, K., & Toldrá, F. (2012). Antihypertensive effect and antioxidant activity of peptide fractions extracted from Spanish dry-cured ham. *Meat Science*, 91(3), 306–311.
- Escudero, E., Mora, L., Fraser, P. D., Aristoy, M.-C., Arihara, K., & Toldrá, F. (2013). Purification and identification of antihypertensive peptides in Spanish dry-cured ham. *Journal of Proteomics*, 78, 499–507.
- Esteve, C., Marina, M. L., & García, M. C. (2015). Novel strategy for the revalorization of olive (*Olea europaea*) residues based on the extraction of bioactive peptides. *Food Chemistry*, 167, 272–280.
- Fakhfakh, N., Ktari, N., Siala, R., & Nasri, M. (2013). Wool-waste valorization: production of protein hydrolysate with high antioxidative potential by fermentation with a new keratinolytic bacterium, *Bacillus pumilus* A1. *Journal of Applied Microbiology*, 115(2), 424–433.
- García, M. C., Endermann, J., González-García, E., & Marina, M. L. (2015). HPLC-Q-TOF-MS Identification of Antioxidant and Antihypertensive Peptides Recovered from Cherry (*Prunus cerasus* L.) Subproducts. *Journal of Agricultural and Food Chemistry*, 63(5), 1514–1520.
- García-mora, P., Martín-martínez, M., Bonache, M. A., González-múniz, R., Peñas, E., Frias, J., & Martínez-, C. (2016). Identification, functional gastrointestinal stability and molecular docking studies of lentil peptides with dual antioxidant and angiotensin I converting enzyme inhibitory activities. *Food Chemistry*. <http://doi.org/10.1016/j.foodchem.2016.10.087>
- Ghribi, A. M., Sila, A., Przybylski, R., Nedjar-Arroume, N., Makhlouf, I., Blecker, C., ... Besbes, S. (2015). Purification and identification of novel antioxidant peptides from enzymatic hydrolysate of chickpea (*Cicer arietinum* L.) protein concentrate. *Journal of Functional Foods*, 12, 516–525.

- 418 Girgih, A. T., He, R., Malomo, S., Offengenden, M., Wu, J., & Aluko, R. E. (2014).
419 Structural and functional characterization of hemp seed (*Cannabis sativa* L.)
420 protein-derived antioxidant and antihypertensive peptides. *Journal of Functional*
421 *Foods*, 6, 384–394.
- 422 Hartmann, R., & Meisel, H. (2007). Food-derived peptides with biological activity:
423 from research to food applications. *Current Opinion in Biotechnology*, 18(2), 163–
424 169.
- 425 He, R., Ju, X., Yuan, J., Wang, L., Girgih, A. T., & Aluko, R. E. (2012). Antioxidant
426 activities of rapeseed peptides produced by solid state fermentation. *Food*
427 *Research International*, 49(1), 432–438.
- 428 Hernández-Ledesma, B., del Mar Contreras, M., & Recio, I. (2011). Antihypertensive
429 peptides: production, bioavailability and incorporation into foods. *Advances in*
430 *Colloid and Interface Science*, 165(1), 23–35.
- 431 Jemil, I., Abdelhedi, O., Mora, L., Nasri, R., Aristoy, M., Jridi, M., ... Nasri, M. (2016).
432 Peptidomic analysis of bioactive peptides in zebra blenny (*Salaria basilisca*)
433 muscle protein hydrolysate exhibiting antimicrobial activity obtained by
434 fermentation with *Bacillus mojavensis* A21. *Process Biochemistry*, 51(12), 2186–
435 2197. <http://doi.org/10.1016/j.procbio.2016.08.021>
- 436 Jemil, I., Jridi, M., Nasri, R., Ktari, N., Salem, R. B. S.-B., Mehiri, M., ... Nasri, M.
437 (2014). Functional, antioxidant and antibacterial properties of protein hydrolysates
438 prepared from fish meat fermented by *Bacillus subtilis* A26. *Process Biochemistry*,
439 49(6), 963–972.
- 440 Jemil, I., Mora, L., Nasri, R., Abdelhedi, O., Aristoy, M.-C., Hajji, M., ... Toldrá, F.
441 (2016). A peptidomic approach for the identification of antioxidant and ACE-
442 inhibitory peptides in sardinelle protein hydrolysates fermented by *Bacillus subtilis*
443 A26 and *Bacillus amyloliquefaciens* An6. *Food Research International*.
444 <http://doi.org/10.1016/j.foodres.2016.08.020>
- 445 Korhonen, H., & Pihlanto, A. (2003). Food-derived bioactive peptides-opportunities for
446 designing future foods. *Current Pharmaceutical Design*, 9(16), 1297–1308.
- 447 Lassoued, I., Mora, L., Barkia, A., Aristoy, M., Nasri, M., Toldrá, F., & others. (2016).
448 Angiotensin I-converting enzyme inhibitory peptides FQPSF and LKYPI identified
449 in *Bacillus subtilis* A26 hydrolysate of thornback ray muscle. *International Journal*
450 *of Food Science & Technology*, 51(7), 1604–1609.
- 451 Lassoued, I., Mora, L., Nasri, R., Jridi, M., Toldrá, F., Aristoy, M.-C., ... Nasri, M.
452 (2015). Characterization and comparative assessment of antioxidant and ACE
453 inhibitory activities of thornback ray gelatin hydrolysates. *Journal of Functional*
454 *Foods*, 13, 225–238.
- 455 Liu, M., Du, M., Zhang, Y., Xu, W., Wang, C., Wang, K., & Zhang, L. (2013).
456 Purification and identification of an ACE inhibitory peptide from walnut protein.
457 *Journal of Agricultural and Food Chemistry*, 61(17), 4097–4100.
- 458 Liu, R., Zheng, W., Li, J., Wang, L., Wu, H., Wang, X., & Shi, L. (2015). Rapid
459 identification of bioactive peptides with antioxidant activity from the enzymatic

460 hydrolysate of *Macraa veneriformis* by UHPLC--Q-TOF mass spectrometry. *Food*
461 *Chemistry*, 167, 484–489.

462 Minkiewicz, P., Dziuba, J., Iwaniak, A., Dziuba, M., & Darewicz, M. (2008). BIOPEP
463 database and other programs for processing bioactive peptide sequences. *Journal*
464 *of AOAC International*, 91(4), 965–980.

465 Moayedi, A., Hashemi, M., & Safari, M. (2015). Valorization of tomato waste proteins
466 through production of antioxidant and antibacterial hydrolysates by proteolytic
467 *Bacillus subtilis*: optimization of fermentation conditions. *Journal of Food Science*
468 *and Technology*, 53(1), 391–400. <http://doi.org/10.1007/s13197-015-1965-2>

469 Moayedi, A., Mora, L., Aristoy, M.-C., Hashemi, M., Safari, M., & Toldrá, F. (2017).
470 ACE-Inhibitory and Antioxidant Activities of Peptide Fragments Obtained from
471 Tomato Processing By-Products Fermented Using *Bacillus subtilis*: Effect of
472 Amino Acid Composition and Peptides Molecular Mass Distribution. *Applied*
473 *Biochemistry and Biotechnology*, 181(1), 48–64.

474 Mora, L., Escudero, E., Fraser, P. D., Aristoy, M.-C., & Toldrá, F. (2014). Proteomic
475 identification of antioxidant peptides from 400 to 2500Da generated in Spanish
476 dry-cured ham contained in a size-exclusion chromatography fraction. *Food*
477 *Research International*, 56, 68–76.

478 Nakamura, Y., Yamamoto, N., Sakai, K., Okubo, A., Yamazaki, S., & Takano, T.
479 (1995). Purification and characterization of angiotensin I-converting enzyme
480 inhibitors from sour milk. *Journal of Dairy Science*, 78(4), 777–783.

481 Niu, L.-Y., Jiang, S.-T., & Pan, L.-J. (2013). Preparation and evaluation of antioxidant
482 activities of peptides obtained from defatted wheat germ by fermentation. *Journal*
483 *of Food Science and Technology*, 50(1), 53–61.

484 Pownall, T. L., Udenigwe, C. C., & Aluko, R. E. (2010). Amino acid composition and
485 antioxidant properties of pea seed (*Pisum sativum* L.) enzymatic protein
486 hydrolysate fractions. *Journal of Agricultural and Food Chemistry*, 58(8), 4712–
487 4718.

488 Sentandreu, M. Á., & Toldrá, F. (2006). A rapid, simple and sensitive fluorescence
489 method for the assay of angiotensin-I converting enzyme. *Food Chemistry*, 97(3),
490 546–554.

491 Shahidi, F., & Zhong, Y. (2008). Bioactive peptides. *Journal of AOAC International*,
492 91(4), 914–931.

493 Udenigwe, C. C., & Aluko, R. E. (2012). Food Protein-Derived Bioactive Peptides:
494 Production, Processing, and Potential Health Benefits. *Journal of Food Science*,
495 77(1), R11–R24.

496 Yamamoto, N., Akino, A., & Takano, T. (1994). Antihypertensive effect of the peptides
497 derived from casein by an extracellular proteinase from *Lactobacillus helveticus*
498 CP790. *Journal of Dairy Science*, 77(4), 917–922.

499 Yang, X., Teng, D., Wang, X., Guan, Q., Mao, R., Hao, Y., & Wang, J. (2016).
500 Enhancement of Nutritional and Antioxidant Properties of Peanut Meal by Bio-
501 modification with *Bacillus licheniformis*. *Applied Biochemistry and Biotechnology*,

180(6), 1227–1242. <http://doi.org/10.1007/s12010-016-2163-z>

Zhang, Y., Liu, J., Lu, X., Zhang, H., Wang, L., Guo, X., ... Qian, H. (2014). Isolation and identification of an antioxidant peptide prepared from fermented peanut meal using *Bacillus Subtilis* fermentation. *International Journal of Food Properties*, 17(6), 1237–1253.

Zheng, Y., Li, Y., Zhang, Y., Ruan, X., & Zhang, R. (2017). Purification , characterization , synthesis , in vitro ACE inhibition and in vivo antihypertensive activity of bioactive peptides derived from oil palm kernel glutelin-2 hydrolysates. *Journal of Functional Foods*, 28, 48–58. <http://doi.org/10.1016/j.jff.2016.11.021>

Fig. 1. Schematic protocol corresponding to preparation, purification and identification of bioactive peptides in this study.

Fig. 2. Size Exclusion Chromatographic fractionation and purification of hydrolysates from fermented tomato waste proteins on Sephadex G-25.

520 Fig. 3. RP-HPLC fractionation and purification of the most active fractions obtained after
521 fractionation using SEC (Sephadex-G25).

523

524

525 Fig. 4. Ferric reducing power of selected synthesized peptides in comparison with synthetic
526 antioxidant BHT.

527

528 **Table 1. Some of peptides containing active domains (according to BioPep data base) identified in this**
529 **study.**

Peptide sequence	Protein source	Calculated molecular mass	Fraction*
VPPAN	Asparaginyl-tRNA synthetase, cytoplasmic 3	496.2645	F1, F2, F3, F4
GQVPP	Pentatricopeptide repeat-containing protein At4g17616	496.2646	F1, F4
VPPKG	Asparaginyl-tRNA synthetase, cytoplasmic 1	496.3009	F1, F4
KDVPP	Probable glycerophosphoryl diester phosphodiesterase 1	554.3064	F1, F3
IGQVPP	Pentatricopeptide repeat-containing protein At4g17616	609.3486	F4
GQVPPQ	Pentatricopeptide repeat-containing protein At4g17616	624.3231	F1
DVPPYM	Probable glycerophosphoryl diester phosphodiesterase 1	720.3153	F1
EIVPPAN	Asparaginyl-tRNA synthetase	738.3912	F1
VPPLYPN	Transcription factor bHLH100	798.4276	F2
QVPPQLLG	Pentatricopeptide repeat-containing protein At4g17616	850.4913	F3
KVPPKGKGT	Asparaginyl-tRNA synthetase	910.56	F1
LKVPPKGKG	Asparaginyl-tRNA synthetase	922.5964	F1
EIVPPATQL	Asparaginyl-tRNA synthetase	966.5386	F3
EIVPPANQLA	Asparaginyl-tRNA synthetase	1050.5709	F2
VPPQLLGHYQH	Pentatricopeptide repeat-containing protein At4g17616	1287.6724	F3
TRKDVPPYMIPVQ	Probable glycerophosphoryl diester phosphodiesterase 1	1542.8228	F3
GDEIVPPANQLAADNLENDG	Asparaginyl-tRNA synthetase, cytoplasmic 3	2050.9443	F3
PHHIA	Homeobox-leucine zipper protein ROC8	573.3023	F1
VPHHI	Homeobox-leucine zipper protein ROC8	601.3336	F3
AIGPHH	Zinc finger CCCH domain-containing protein 66	630.3238	F4
PAIGPHHLG	Zinc finger CCCH domain-containing protein 66	897.4821	F1
VALGVPHH	Homeobox-leucine zipper protein ROC8	828.4606	F4
LVALGVPHHIA	Homeobox-leucine zipper protein ROC8	1125.6659	F4
SAAAPAIGPHHL	Zinc finger CCCH domain-containing protein 66	1140.604	F4
SHYFYQCY	Pectinesterase QRT1	1109.4277	F2
YFYQC	Pectinesterase QRT1	722.2734	F2
CPGLP	(S)-N-methylcoclaurine 3~-hydroxylase isozyme 1	485.2308	F1
KPGLP	Asparaginyl-tRNA synthetase, cytoplasmic 3	510.3166	F2
PGLPK	Asparaginyl-tRNA synthetase, cytoplasmic 3	510.3166	F1
LDPGL	Dof zinc finger protein DOF3.3	513.2799	F2
DPGLQ	Dof zinc finger protein DOF3.3	528.2544	F3
RPGLP	Asparaginyl-tRNA synthetase, cytoplasmic 1	538.3227	F1, F3
GPGLVT	Serine/threonine-protein phosphatase BSL1 homolog	542.3064	F1
PGLQAN	Dof zinc finger protein DOF3.3	598.3075	F1
ICPGLP	(S)-N-methylcoclaurine 3~-hydroxylase isozyme 1 (Fragment)	598.3149	F2
VDLPGL	15.4 kDa class V heat shock protein	612.3483	F3
DLPGLT	Dynamin-related protein 1C	614.3275	F3
PGLPKK	Asparaginyl-tRNA synthetase, cytoplasmic 1	638.4115	F2
VDLPGLR	15.4 kDa class V heat shock protein	768.4494	F3
CPGLPIAN	(S)-N-methylcoclaurine 3~-hydroxylase isozyme 1	783.3949	F1
PGLPKKDG	Asparaginyl-tRNA synthetase, cytoplasmic 1	810.4599	F3

PNARGPGLV	Serine/threonine-protein phosphatase BSL1 homolog	879.4926	F1
DLPGGTKVA	Dynamin-related protein	912.528	F2
DPGLQANTR	Dof zinc finger protein DOF3.3	970.4832	F1
RLRLKPGLP	Asparaginyl-tRNA synthetase, cytoplasmic 3	1048.6869	F3
DPGLQANTRV	Dof zinc finger protein DOF3.3	1069.5516	F1
GRRICPGLPI	(S)-N-methylcoclaurine 3~-hydroxylase isozyme 1 (Fragment)	1080.6226	F1
HTFSVDLPGL	15.4 kDa class V heat shock protein	1084.5553	F3
AARLDPGLQAN	Dof zinc finger protein DOF3.3	1124.5938	F4
IDLPGLTKVAV	Dynamin-related protein 1C	1124.6805	F1
LRLKPGLPKNDG	Asparaginyl-tRNA synthetase	1306.7721	F2
RPGLPKKDGND	Asparaginyl-tRNA synthetase	1308.715	F1
SPDSHTFSVDLPGL	15.4 kDa class V heat shock protein	1470.6991	F1
ICPGLPIANQFIALLV	(S)-N-methylcoclaurine 3~-hydroxylase isozyme 1	1680.9637	F3
DGVVYY	Putative F-box protein At5g52610	714.3225	F4
CIDGVVYYG	Putative F-box protein At5g52610	987.4372	F1
DGVVYYGAQT	Putative F-box protein At5g52610	1071.4873	F1
ADVTEPPLPP	Probable glycerophosphoryl diester phosphodiesterase 1	1034.5284	F3
IHPLPPPVNSP	Serine/threonine-protein phosphatase BSL1 homolog	1166.6448	F4
GKPLP	Phosphoenolpyruvate carboxylase 4	510.3166	F3
EPPLP	Probable glycerophosphoryl diester phosphodiesterase 1	551.2955	F3
HPLPP	Serine/threonine-protein phosphatase BSL1 homolog	559.3118	F4
EPPLPP	Probable glycerophosphoryl diester phosphodiesterase 1	648.3483	F4
PLPPPVNSP	Serine/threonine-protein phosphatase BSL1 homolog	916.5018	F4
FASGVLVPK	Probable glycerophosphoryl diester phosphodiesterase 1	916.5382	F1
VLVPK	Probable glycerophosphoryl diester phosphodiesterase 1	554.3792	F1
PKLPPVKK	Protein OSB2, chloroplastic	905.6062	F3
ASLLPPY	Zein-alpha PZ19.1 (Fragment)	759.4167	F2
GFYPPP	Dof zinc finger protein DOF3.3	676.3221	F1
PGVPWP	Dof zinc finger protein DOF3.3	651.3381	F1

530

*: Fraction number from RP-HPLC purification.

531

532 **Table 2.** ACE inhibitory and DPPH scavenging activities of selected synthesized peptides.

Peptide sequence	Mass (Da)	Fraction (from HPLC)	ACE inhibitory (IC ₅₀ , mM)	DPPH scavenging (at 0.4 mM)
VLVPK	554.3792	[F1]	0.365	6%
KDVPP	554.3064	[F1]	1.231	3%
GQVPP	496.2646	[F1] [F4]	0.360	97%
PKLPPVKK	905.6062	[F3]	0.346	5%
PGLPKK	638.4115	[F2]	0.032	n.a
ASLLPPY	759.4167	[F2]	0.334	n.a
VALGVPHH	828.4606	[F4]	n.a*	n.a
DGVVYY	714.3225	[F1],[F4]	0.002	16%
IVEYF	669.3374	[F1]	0.138	5%
GFYPPP	676.3221	[F1]	0.032	5%
DVPPYM	720.3153	[F1]	0.581	4%
VPPLYPN	798.4276	[F2]	0.056	n.a
PGVPWP	651.3381	[F1]	0.041	7%

533 *No activity.

534