ACE-inhibitory and antioxidant activities of peptide fragments obtained from tomato processing by-products fermented using *Bacillus subtilis*: Effect of Amino Acid Composition and Peptides Molecular Mass Distribution

Ali Moayedi[1], Leticia Mora[2], Maria C. Aristoy[2], Maryam Hashemi[3], Mohammad Safari[4], Fidel Toldrá*[2]

[1]Department of Food Science and Technology, Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Iran
[2]Instituto de Agroquimica y Technologia de Alimentos (CSIC), Avenue Agustin Escardino, Paterna, Valencia, Spain
[3]Department of Microbial Biotechnology and Biosafety, Agricultural Biotechnology Research Institute of Iran (ABRII), AREEO, P.O. Box 3135933151, Karaj, Iran
[4]Department of Food Science, Engineering and Technology, University of Tehran, Karaj, Iran

*Corresponding author address: Fidel Toldrá (ftoldra@iata.csic.es) Tel: +34963900022 ext. 2112; Fax: +34963636301

Abstract

The effects of amino acid composition and peptide molecular mass on ACE-inhibitory and antioxidant activities of protein fragments obtained from tomato waste fermented using *Bacillus subtilis* were evaluated. The addition of *B. subtilis* increased the relative amounts of aromatic and positively-charged amino acids which have been described to influence the biological activities of peptide fragments. IC_{50} values of hydrolysates for ACE-inhibitory and 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) scavenging activities were found to be 1.5 mg/mL and 8.2 mg/mL, respectively. Size-exclusion chromatography (SEC) pattern of the hydrolysate indicated the breakdown of parent proteins to smaller peptides with molecular weights mainly below 1400 Da. MALDI-TOF mass spectrometry analysis revealed that the highest ACE-inhibitory activity was due to peptides showing molecular mass range 500 - 800 Da, while the most active antioxidant peptides were found to be mainly at the two different peptide weight ranges 500 – 800 Da and 1200 – 1500 Da.

Keywords: Fermentation, Bioactive peptides, Size Exclusion Chromatography, MALDI-TOF

Introduction

Recent studies show that twenty percent of world adult population suffers from hypertension and diseases like stroke, coronary heart disease (CHD), kidney dysfunction, and myocardial infarction [1]. Hypertension is mainly regulated by the renin-angiotensin system. The angiotensin-1 converting enzyme (ACE) catalyzes the conversion of angiotensin-1, a vasodilator, into angiotensin-2, a vasoconstrictor agent [2]. Also electrolyte homeostasis, an effective factor in blood pressure, is associated with the action of ACE [3]. Therefore, those compounds able to inhibit ACE activity have been described to result helpful to control hypertension effects. In this sense, several synthetic drugs such as captopril, enalapril, alacepril or lisinopril have been delivered to treat myocardial infraction, hypertension and other cardio-related diseases [4]. However, some side effects like inflammatory response, dry cough, taste disturbances or angioneurotic edema have been related to the use of such drugs in some patients [5]. For this reason, different studies have been carried out to find alternative ACE inhibitors, from which food protein derived peptides and hydrolysates have gained great attention.

The different oxidation mechanisms occurring during food processing or storage lead to the formation of free radicals and reactive oxygen species (ROS) [6, 7]. ROS and free radicals have been described to affect food by decreasing its nutritional quality and producing off-flavors, but also may affect the human metabolism causing DNA damage, tissue injuries and several diseases in the body [7]. Synthetic antioxidants such as t-butylhydroquinone (TBHQ), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate have been traditionally used in food products. However, because of recent concerns about the safety of synthetic antioxidants, an increasing attention has been paid to natural antioxidants especially derived from natural sources such as protein hydrolysates [8].

Protein hydrolysates and peptides showing antioxidant and ACE inhibitory activities have been described to be generated from different sources of plant and animal proteins. More recently, food processing wastes and by-products have been of great interest to be considered for the production of antioxidant and ACE-inhibitory peptides [5, 9, 10]. In this sense, the tomato processing industry generates 7-7.5% of solid wastes. A total of 70-72 % of tomato waste is pomace containing around 44% seeds (based on dried pomace). Protein constitutes 28% of tomato seed weight which is rich in globulin (60-70 %) [11]. So it is expected that a considerable amount of high protein by-products could be generated from the tomato processing industries all over the world. However, there are limited studies investigating the potential of tomato waste proteins to produce added-value products.

In this study, a submerged fermentation using the proteolytic strain *Bacillus subtilis* A14h to generate bioactive peptides from tomato seed proteins was employed. Then, the effects of changes in relative amounts of amino acids (during fermentation) and molecular mass distribution of the generated peptides were investigated. ACE-inhibitory and antioxidant activities obtained in a size-exclusion chromatographic separation were evaluated and MALDI-TOF mass spectrometry analysis was employed to monitor molecular mass distribution in each active peptide fraction precisely.

MATERIAL AND METHODS

Reagents and Chemicals

2, 2′-diphenyl-1-picrylhydrazyl (DPPH) and ACE enzyme (from rabbit lung) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Abz-Gly-p-nitro-phe-pro-OH trifluoroacetate salt used as substrate was purchased from Bachem AG. (Bubendorf, Switzerland). Microbial culture media were purchased from Sharlau (Barcelona, Spain).

Acetonitrile, hydrochloric acid 37%, and trifluoroacetic acid (TFA) were of HPLC grade (Scharlau). All other chemicals were of analytical grade (Scharlau).

Tomato seed meal preparation

Tomato pomace containing skins and seeds was provided by Golgashte Shirin plant (Ghazvin, Iran). The seeds were separated and sun-dried after removing the skin by immersing pomace in water, and then ground in a blender. The resultant powder was defatted using Soxhlet method with n-hexane as solvent, dried again, and ground. The powdered tomato seed meal (TSM) was stored in the refrigerator until use.

Bacterial culture

The cells of *B. subtilis* A14h which displayed high protease activity in a previous study [12] were obtained from the microbial bank of the Agricultural Biotechnology Research Institute of Iran (ABRII). After activation on nutrient agar, cells were transferred into a fresh nutrient broth medium (3% peptone, 0.5% beef extract, 0.5% NaCl, and 0.1% glucose) and allowed to grow at 30 °C for 18 h at 160 rpm. Then, at the beginning of the logarithmic growth phase, the cells centrifuged at 3800 rpm (Suprema 25, TOMY, Japan) for 20 min at 15 °C, and re-suspended in sterile physiological serum before being added in the fermentation media.

Fermentation and hydrolysis

In order to obtain TSM hydrolysates, a submerged fermentation system with 250 mL Erlenmeyer flasks (working volume 25 mL) containing TSM as substrate was applied as previously described by Moayedi *et al*. [12]. The fermentation medium was comprised of TSM (6%, w/v), K_2HPO_4 (0.05%, w/v), $MgSO_4$ (0.01%, w/v) and $CaCO_3$ (0.16% w/v). Then, the medium was inoculated (2%, v/v) with freshly prepared bacterial cells (1×10^8 CFU/mL) and incubated for 24 h at 37 °C under agitation conditions. To stop the fermentation process

and inactivate proteolytic enzymes, the whole sample was heated in boiling water for 15 min and then centrifuged at 12000 rpm (Suprema 25, TOMY, Japan) for 10 min. The supernatant was collected, lyophilized, and kept at -20 °C until use. The mentioned lyophilized powder is hereinafter referred to as TSMH (tomato seed meal hydrolysate). The same process except for inoculating with bacterial cells was done to prepare the control slurry.

Molecular mass distribution

Size exclusion chromatography (SEC) system was used to estimate the molecular mass distribution of protein and peptide fragments in TSMH and control slurry. For this purpose, lyophilized TSMH and control slurry were completely dissolved in bi-distilled water (25 mg/mL), the solution was centrifuged at 10000×g for 10 min and the supernatant was filtered through 0.22 µm syringe filter. Five mL of final clear solution was loaded onto a Sephadex G-50 column. HCl 0.01 N at a flow rate of 15 mL/h was used as the eluent. Fractions of 5 mL were collected at 20 min intervals by using an automated collector and the absorbance was measured at 214, 254 and 280 nm using an UV-visible spectrophotometer (Agilent 8453 UV spectrophotometer, Agilent Tech., Palo Alto, CA, USA). The column was calibrated by loading standard gel filtration molecular weight markers, bovine serum albumin (66,000 Da), Chymotrypsinogen (25,600 Da), Cytochrome C (13,000), Aprotinin (6,511.44 Da), Bacitracin (1,422.69 Da), and Tyrosine (180 Da).

In order to evaluate the molecular weight distribution of peptide fragments and relate it to the ACE-inhibitory and antioxidant activity, groups of fractions from an elution volume of 100 mL were pooled together (50 mL each), lyophilized and stored. New fractions were named F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F11, and F12, corresponding to elution volumes of 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 400- 450, 450-500, 500-550, 550-600, 600-650, and 650-700 mL, respectively.

Amino acid analysis

Determination of amino acid composition in TSMH and control was done according to the Pico Tag procedure [13]. Briefly, the lyophilized sample was submitted to hydrolysis using HCl 6N at 110 °C for 22 h under nitrogen atmosphere in evacuated sealed tubes. After vacuum drying and derivitizing with phenylisothiocyanate, 20 µL of each sample, previously dissolved in sodium phosphate buffer (5 mM K_2HPO_4, pH 7.4, containing 5% acetonitrile), was injected to an Agilent 1100 HPLC instrument (Agilent Tech.) equipped with an analytical Pico Tag column (300 x 4 mm, 5 µm) from Waters (Wexford, Ireland). Amino acid composition was reported as relative percentage of each amino acid to the total.

MALDI-TOF mass spectrometry analysis

The analysis was done in a 5800 MALDI-ToF/ToF instrument (ABSciex, CA, USA) in positive reflectron mode (3000 shots every position) in two different ranges: 150-850 Da and 850-3500 Da; the laser intensity was manually adjusted to maximize the S/N ratio. Plate model and acquisition method were calibrated by ABSCIEX calibration mixture (des-Arg1-Bradykinin at 1fmol/µL; Angiotensin I at 2 fmol/µL; Glu1-Fibrinopeptide B at 1.3 fmol/µL; ACTH (1–17 clip) at 2 fmol/µL; ACTH (18–39 clip) at 5 fmol/µL; and ACTH (7–38 clip) at 3 fmol/µL) in 13 positions. Dried hydrolysates were dissolved in 5% ACN; 0.1% TFA, and 1 µL of every sample was directly spotted on 10 positions in the MALDI plate and allowed to air dry. Once dried, 0.5 µL of matrix solution (5 mg/mL of α-Cyano-4-hydroxycinnamic acid (CHCA) in 0.1% TFA-ACN/H_2O (7:3, v/v) was spotted. The data was analyzed by using mMass software (http://www.mmass.org/).

Determination of ACE inhibitory activities

The ACE inhibitory activities of TSMH and control were measured according to the fluorescence-based method previously described by Sentandreu and Toldrá [2]. In this assay, the internally quenched fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe-(NO2)-Pro) is hydrolyzed by ACE to release the fluorescent product o-aminobenzoylglycine (Abz-Gly). 50 µL of 3 mU/mL ACE preparation in Tris-base buffer (150 mM, pH 8.3) was added to 50µL of sample, and the mixture was pre-incubated at 37 °C for 10 min. Then, 200 µL of 150 mM Tris–HCl buffer (pH 8.3) containing 1.125 M NaCl and 10 mM Abz-Gly-Phe-(NO2)-Pro was added and the reaction mixture was incubated for 60 min at 37 °C. The fluorescence intensity was measured using excitation and emission wavelengths of 355 and 405 nm, respectively. Bidistilled water was used as negative control whereas captopril (0.1mg/mL) was used as positive control in the assay. ACE inhibitory activity was expressed as percentage and measurements were done in triplicate (n=3). IC_{50} value as the amount of peptide or hydrolysate required for inhibiting 50% of ACE activity was also calculated.

Determination of antioxidant activities

DPPH scavenging activity

DPPH scavenging activity analysis was carried out according to Jemil *et al.* [14] with minor modifications. Briefly, 190 µL of sample was mixed with 220 µL of ethanol and 95 µL DPPH solution (2%, w/v in ethanol). The mixture was kept for 60 min in the dark and absorbance was measured at 517 nm. The negative control was prepared using distilled water instead of the sample and BHT was used as positive control. The percentage of DPPH scavenging activity was calculated according to the following equation:

$$\%\text{DPPH scavenging activity} = \left(1 - \frac{As}{Ac}\right) \times 100$$

Where, As is the absorbance of sample and Ac is the absorbance of the control. Antioxidant activity was measured in TSMH and control samples in triplicate.

Reducing power capacity

The reducing power (on Fe (III)) of the supernatant was measured similar to the method described by Yildrim *et al.* (2001) [15] with minor changes. Briefly, 100 µL of sample was mixed with 250 µL of 0.2M potassium phosphate buffer (pH 6.6) and 250 µL of 1% (w/v) potassium ferricyanide and incubated for 30 min at 50°C. Then, 250 µL of 10% TCA was added, mixed, and centrifuged at 12000 rpm for 10 min. After that, 250 µL of the supernatant were taken and 250 µL of distilled water and 50 µL of 0.1% (w/v) ferrous chloride were added. After standing the mixture at room temperature for 10 min, the absorbance was measured at 700 nm. Antioxidant activity was measured in TSMH and control samples in triplicate.

Statistical analysis

Data was statistically analyzed using Statistical Analysis System (SAS) v9.1 software (SAS Institute, Inc., Cary, NC). Significant differences between the mean values were determined using the Duncan Multiple Range Test ($p \leq 0.05$).

Results and Discussion

ACE-inhibitory activity of TSMH

In this study, *in vitro* ACE inhibitory capacities of control and TSMH were evaluated at different concentrations. As it is shown in Fig. 1a, ACE inhibitory activities of both TSMH and control slurry increased as concentration increased from 1 to 10 mg/mL. At all tested concentrations, ACE inhibitory activity of TSMH was significantly higher than that for control, showing values more than 4 and 2.5 folds higher at concentrations of 2 and 4 mg/mL,

respectively. This finding is in accordance with several previous reports on the positive effects of fermentation on ACE-inhibitory activity in plant and animal protein extracts. Fontoura *et al*. [16] and Hu *et al*. [17] reported that fermentation with keratinolytic strain *Chrysobacterium* Kr6 and *B. subtilis* increased the ACE inhibitory activities of soluble supernatants in chicken feather and black soybean hydrolysates, respectively [16, 17]. In contrast, Limon *et al.* [18] observed that fermentation of kidney bean with *B. subtilis* was followed by a considerable decline in ACE inhibitory activity of supernatant in comparison with unfermented group. This inconsistence observed in different studies may be related to differences in microbial starter type, substrate, and fermentation conditions which might affect ACE inhibitory properties of resulting products.

On the other hand, the IC_{50} value of ACE inhibitory activity of TSMH in the current study was found to be 1.5 mg/mL which was 6 times lower than that for control. This IC_{50} level was comparable with the IC_{50} values reported for Goby fish protein hydrolysate [19], but lower than those for rice bran protein hydrolysate (5.2 mg/mL) and bromelain-hydrolyzed trevally protein (1.99 to 3.34 mg/mL) [20]. Sheep whey protein hydrolysate obtained after hydrolysis using partially purified protease from *B. subtilis* showed 57% ACE inhibitory activity at 50 mg/mL concentration [21]. However, Ambigaipalan *et al*. [5] reported IC_{50} equal to 0.53 mg/mL for date seed protein hydrolysate of *Phoenix dactylifera* obtained under the best hydrolysis conditions and Esteve *et al* [9] reported IC_{50} values of 0.35 mg/mL for olive seed hydrolysate, both in ACE inhibitory assay.

Our results indicated that ACE inhibitory sequences were probably encrypted in TSM proteins and released during fermentation by employing a suitable proteolytic system. Also, the results reflected the capabilities of *B. subtilis* proteases to hydrolyze TSM proteins and release such bioactive peptides. Differences between ACE inhibitory activity of TSMH and

that of control might be attributed to the presence of peptide fragments with suitable amino acid compositions and sequences.

Antioxidant capacities of TSMH

DPPH radical scavenging activity of TSMH showed also a dose-dependent effect that increases with the concentration (Fig. 1b), reaching a value of 57% at 10 mg/mL. The trend of DPPH scavenging activity of TSMH was in agreement with previously reported studies in which this activity was also dose-dependent [22, 23]. The IC_{50} value for TSMH sample was 8.2 mg/mL in DPPH scavenging activity. This value was higher than the IC_{50} value reported by He *et al*. [24] in fermented rape seed using *B. subtilis* (165 µg/mL), but close to the IC_{50} value observed by Jemil *et al*. [14] in fermented sardine protein using *B. subtilis* A26 (6 mg/mL approximately).

Reducing power assay is considered as a direct method for measuring antioxidant activity and reflects electron-donating capacity of an antioxidant compound. Therefore, the compounds indicating ferric reducing activity are able to reduce some oxidized compound during peroxidation process. Those protein hydrolysates showing higher values of absorbance in reducing power assay have better capacity to donate electrons. Reducing power of TSMH (Fig. 1c) in this study ranged from 0.3 to 0.77 (as expressed by absorbance intensity at 700 nm) at concentrations from 2 to 10 mg/mL which was comparable with previously published reducing power values of sardine protein hydrolysate [14]. On the other hand, reducing power of TSMH in this study at 10 mg/mL (absorbance of 0.77) was also comparable to the reducing power observed in ovine casein hydrolysate at 15 mg/mL [25].

TSMH showed higher antioxidant activity than control slurry. In general, antioxidant activity of protein hydrolysates is referred to their electron donating properties. Therefore, differences between TSMH and control samples in the results obtained using reducing power and DPPH

scavenging methodologies is probably due to the presence of peptides generated during the fermentation process showing specific amino acid composition and sequences.

Amino acid composition

In order to know which is the contribution of *B. subtilis* A14h to the amino acids profile the comparison between the amino acid relative composition of control and TSMH sample was done, and the result is shown in Table 1. The relative composition of amino acids is different between samples, but Glx (glutamic acid + glutamine) were found to be the most abundant amino acids in both. The *B. subtilis* addition significantly increased relative amount of tyrosine, phenylalanine and lysine in TSMH in comparison to the control (Table 1). In fact, a 2-fold higher percentage of aromatic amino acids in TSMH (11.27%) in comparison to control slurry (4.49%) was observed. In particular, phenylalanine percentage showed a marked increase from 3.51% in control to 7.30% in hydrolysate (TSMH). In addition, arginine and lysine percentages increased in the hydrolysate which resulted in a higher amount of total positively charged amino acids from 12.17% (in control) to 15.75% (in TSMH).

Antioxidant and ACE inhibitory activities of peptides and protein hydrolysates are influenced by factors like protein source, hydrolysis conditions, degree of hydrolysis, molecular mass, and amino acid composition as well as the position of amino acids in the peptide sequences. In this sense, the correlation between specific amino acid groups and biological activities has been described in different studies about pea and apricot almond protein hydrolysates [23, 26].

It has been shown that hydrophobic amino acid residues like leucine, valine, alanine, tyrosine, phenylalanine, or tryptophan, can act as competitive ACE-inhibitors as they preferably bind the catalytic sites of ACE [5]. In particular, it has been described that the

281 presence of phenylalanine or other aromatic amino acids at each of the three positions closest
282 to the C-terminal is the most suitable position [5], whereby also hydrophobic amino acids
283 such as proline were more active. Moreover, most potent antihypertensive peptides contain
284 positively charged amino acids like lysine and arginine at the C-terminal position [27].
285 Alkaline protease enzymes, one of the major groups of proteases produced by *B. subtilis*
286 strains, show good specificity to aromatic amino acid residues including tyrosine,
287 phenylalanine and tryptophan [28], so it was expected that peptide fragments generated from
288 the fermentation with *B. subtilis* could be rich in aromatic amino acid residues at their C-
289 terminal positions. This matter together with an enhance in the content of positively charged
290 amino acids in TSMH (shown in Table 1) could increase the chance of generation of peptides
291 rich in aromatic residues as well as lysine and arginine in their structure which is an
292 important factor that influence their potential as ACE inhibitory peptides.

293 Additionally, ACE inhibitory peptides have been reported to be short peptides within 2-12
294 amino acid residues [9]. The fact that *B. subtilis* is able to produce various proteases in
295 fermentation medium [29] results in the generation of a wide range of peptides showing ACE
296 inhibitory activity. In fact, results reported by Ambigaipalan *et al*. [5] showed that protein
297 hydrolysates obtained from a combination of proteases displayed higher ACE inhibitory
298 activities than those prepared by the action of individual proteases, at the same degree of
299 hydrolysis.

300 The presence of hydrophobic amino acids and at least one residue of histidine, phenylalanine,
301 tryptophan or tyrosine in peptide structure have been found to affect antioxidant activity of
302 protein hydrolysates [30]. It is in accordance with Zhang *et al*. [31] who reported that
303 rapeseed peptide fractions with higher concentrations of hydrophobic amino acids showed
304 better reducing power activity. Thus, amino acid residues with aromatic ring structure

(phenylalanine, tyrosine, and tryptophan) can donate electron to electron deficient compound, and by this mechanism contribute to antioxidant activity [7].

Molecular mass distribution after Size-Exclusion Chromatography (SEC)

As it has been previously described, the antioxidant and ACE inhibitory activity is closely related to the molecular mass of peptides. Bioactive peptides with lower molecular mass may have higher chance to cross the intestinal barrier and exert the biological effects. In fact, main antioxidant and ACE-inhibitory peptides described usually contain 2-20 amino acid residues per molecule. Thus, in order to better understand the role of molecular mass distribution affecting antioxidant and antihypertensive capacities of TSMH and to evaluate the changes of different protein fractions during fermentation, TSMH and control slurry were individually fractionated using SEC. SEC profiles of both TSMH and control slurry using a Sephadex G-50 column are presented in Fig. 2. Control sample showed six main peaks with the major peak in the molecular range of 6500 – 13000 Da (fractions from 35 to 60) according to the analysed standards (see Fig 2.c). The first peak includes proteins having a molecular mass of 13000 – 66000 Da. SEC profile also showed an important peak corresponding to low molecular weight fragments since they are eluting later than the bacitracin standard (1423 Da). Regarding TSMH sample, SEC profile was different from control showing two main peaks eluting later than the bacitracin standard (1423 Da). Comparing both SEC patterns showed in Fig. 2, it could be concluded that hydrolytic activities of proteases produced by *B. subtilis* during fermentation efficiently enhanced the fragmentation of high molecular mass proteins low molecular mass peptides mainly less than 1423 Da. TSMH peak corresponding to fractions between number 116 and 135 mainly contains free amino acids. In this sense, different *B. subtilis* strains have shown to produce a mixture of endo-peptidases and exopeptidases [29].

Several researchers have fractionated protein hydrolysates by using ultrafiltration [32] or SEC [33, 34] and then investigated the effects of molecular size on biological activities of resulting fractions. However, only few studies are focused on fermentation products. Regarding this, from 100 mL elution volume, the collected fractions were grouped (50 mL each), lyophilized, and stored as new fractions. The results of bioactivities for the different fractions obtained in TSMH and control samples are shown in Fig. 3.

ACE inhibitory activities started to be significant from fraction F7, and found to be 9 and 3 folds higher in F7 and F8 of TSMH than control, respectively (Fig. 3a). In fact, the highest values of antioxidant and ACE inhibitory activities in both TSMH and control were detected in fraction F8, which corresponds to a molecular weight area below 1423 Da. In general, it is believed that those peptide fragments showing low molecular mass display stronger ACE inhibitory activities [19, 24, 32, 35, 36]. In the current study, fractions F10, F11, and F12 showed lower ACE inhibitory activity in comparison to fractions F8 and F9, indicating that lower molecular weights not always bring about higher activities. Ruiz-Ruiz *et al.* [32] observed that bean peptide fractions with molecular mass 1.1 – 1.3 kDa showed the highest activity values in ACE-inhibitory assay.

Regarding the effect of peptide size on antioxidant activity, different results were observed depending on the type of antioxidant analysis. DPPH scavenging assay showed the highest antioxidant percentage in fractions F8 and F10 with similar results, whereas the peak value for ferric reducing power assay was observed only on F8. In all cases, TSMH indicated higher antioxidant activity than did control. In this sense, some researchers reported that molecular mass below 3000 Da was the most effective size [37], whereas others suggested 1000 to 3000 Da [38], and even below 1500 Da as the most active sequence sizes [39]. Wu *et al.* [40] observed that mackerel hydrolysate fractions containing peptides with approximately 1400 Da displayed higher protective effect against lipid oxidation in comparison to fractions

with molecular weights between 900 and 200 Da. In addition, in the study performed by Cheung *et al.* [34] it was observed that the most effective molecular mass range varied according to the protease type used to produce Pacific hake protein hydrolysate. The difference in effective molecular mass reported in different studies, and the relation between effective molecular mass and protease type, indicates that amino acid sequence might play a more important role in bioactivity of peptides and protein hydrolysates than molecular mass.

The last fractions of SEC separation (F10-F12 in Figure 3) mainly contain free amino acids. Free amino acids have been reported to have lower bioactivities than small peptides [27]. The fact that previous studies reported lower biological activities in high molecular mass proteins and free amino acids than those detected in small peptides could explain the higher ACE-inhibitory and antioxidant values observed in TSMH in comparison to control sample.

MALDI-TOF mass spectrometry analysis

The results of MALDI-TOF analysis of different active fractions from SEC fractionation of TSMH and control slurry are shown in Fig. 4 and Fig. 5 including *m/z* ranges of 150-850 and 850-3000, respectively. In this respect, fractions F7, F8, F9 and F10 in SEC profile that showed the highest ACE and antioxidant activities correspond to F7-H, F8-H, F9-H and F10-H (for TSMH) and F7-C, FF8-C, F9-C and F10-C (for control slurry), respectively. This method has been widely applied to confirm the extent of hydrolysis and to determine molecular weight distribution in protein hydrolysates [8, 26]. In fact, fractions F10-H and F10-C (the late fractions in SEC profile) showed a higher amount and ion intensity of peptides with molecular weights below 500 Da than those in other groups. In contrast, the number of peptides in the range from 900 to 2700 Da was found to be higher in F7-H. Moreover, MALDI-TOF spectra confirmed higher amount of peptides in TSMH fractions compared to control slurry fractions. Such difference was more evident in the range of 500 –

850 Da for F8-H, F9-H and F10-H; 1200 – 1500 Da for F9-H and F10-H; and 700 – 850 and 1400 – 2000 Da for F7-H in comparison to the control. Small peptides and free amino acids in control might have been released during heating treatment in sterilization process. These fragments were concentrated in molecular weight ranges below 500 Da, especially in F10-C.

Considering the observed ACE-inhibitory activities in F8 as well as the evidences obtained from MALDI-TOF analysis of TSMH fractions and control, it might be concluded that the most active ACE-inhibitory peptides from TSMH in the current study show molecular weight ranges from 500 to 850 Da. However, regarding antioxidant peptides, in addition to 500 to 850 Da, the peptide molecular range from 1200 to 1500 Da was also very effective.

Conclusions

Protein-rich tomato waste was fermented using *B. subtilis* A14h, and the effects of molecular weight and amino acid composition on ACE-inhibitory and antioxidant activities of resulting peptide fragments were evaluated. The results confirmed the previous finding that both amino acid composition and molecular weight play an important role in antioxidant and antihypertensive potential of bioactive peptides generated during protein hydrolysis. Peptides with molecular weight ranges 500-850 Da were found to be very active in both ACE-inhibitory and antioxidant assays. However, the antioxidant activity was not restricted to this mentioned range, and peptides with molecular weights 1200-1500 Da showed also comparable activities. Future *in vivo* studies are suggested to confirm the relation between amino acid composition and molecular weight and bioactivities of peptide fractions.

Acknowledgements

This work was funded by Ministry of Science, Research and Technology of Iran and the Emerging Research Group Grant from Generalitat Valenciana of Spain (GV/2015/138) and JAEDOC-CSIC postdoctoral contract of L.M. cofunded by the European Social Found are

acknowledged. MALDI-TOF analysis was carried out by in the SCSIE University of Valencia Proteomics Unit (Spain), a member of ISCIII ProteoRed Proteomics Platform. Authors would like to thank the helpful support of Carolina Diaz Noriega as technician.

COMPLIANCE WITH ETHICAL STANDARDS

Funding:

This study was funded by Grant of Ministry of Science, Research and Technology of Iran and Emerging Research Group Grant from Generalitat Valenciana in Spain (GV/2015/138). Also a JAEDOC-CSIC postdoctoral contract cofunded by European Social Fund to Dr. Mora.

Conflicts of interest

Author Ali Moayedi declares that he has no conflict of interest. Author Leticia Mora declares that she has no conflict of interest. Author M-Concepción Aristoy declares that she has no conflict of interest. Author Maryam Hashemi declares that she has no conflict of interest. Author Mohammad Safari declares that he has no conflict of interest. Author Fidel Toldrá declares that he has no conflict of interest.

Ethical aproval

This article does not contain any studies with human participants or animals performed by any of the authors.

References

1. Li, Y., Zhou, J., Huang, K., Sun, Y., & Zeng, X. (2012). Purification of a novel angiotensin I-converting enzyme (ACE) inhibitory peptide with an antihypertensive effect from loach (Misgurnus anguillicaudatus). *Journal of agricultural and food chemistry*, *60*(5), 1320–1325.

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

3. Tsai, J.-S., Chen, T.-J., Pan, B. S., Gong, S.-D., & Chung, M.-Y. (2008). Antihypertensive effect of bioactive peptides produced by protease-facilitated lactic acid fermentation of milk. *Food Chemistry*, *106*(2), 552–558.

4. Kleekayai, T., Harnedy, P. A., O'Keeffe, M. B., Poyarkov, A. A., CunhaNeves, A., Suntornsuk, W., & FitzGerald, R. J. (2015). Extraction of antioxidant and ACE inhibitory peptides from Thai traditional fermented shrimp pastes. *Food Chemistry*, *176*, 441–447.

5. 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*, *18*, 1125-1137.

6. Samaranayaka, A. G. P., & Li-Chan, E. C. Y. (2011). Food-derived peptidic antioxidants: A review of their production, assessment, and potential applications. *Journal of functional foods*, *3*(4), 229–254.

7. Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: a review. *Peptides*, *31*(10), 1949–1956.

8. Lassoued, I., Mora, L., Nasri, R., Jridi, M., Toldrá, F., Aristoy, M.-C., … Nasri, M. (2015). Characterization and comparative assessment of antioxidant and ACE inhibitory activities of thornback ray gelatin hydrolysates. *Journal of Functional Foods*, *13*, 225–238.

9. 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.

10. 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.

11. Sogi, D. S., Arora, M. S., Garg, S. K., & Bawa, A. S. (2002). Fractionation and electrophoresis of tomato waste seed proteins. *Food Chemistry*, *76*(4), 449–454.

12. Moayedi, A., Hashemi, M., & Safari, M. (2016). Valorization of tomato waste proteins through production of antioxidant and antibacterial hydrolysates by proteolytic Bacillus subtilis: optimization of fermentation conditions. *Journal of Food Science and Technology*, *53*(1), 391–400.

13. Aristoy, M. C., & Toldra, F. (1991). Deproteinization techniques for HPLC amino acid analysis in fresh pork muscle and dry-cured ham. *Journal of agricultural and food chemistry*, *39*(10), 1792–1795.

14. Jemil, I., Jridi, M., Nasri, R., Ktari, N., Salem, R. B. S.-B., Mehiri, M., … Nasri, M. (2014). Functional, antioxidant and antibacterial properties of protein hydrolysates prepared from fish meat fermented by Bacillus subtilis A26. *Process Biochemistry*, *49*(6), 963–972.

15. Yildirim, A., Mavi, A., & Kara, A. A. (2001). Determination of antioxidant and antimicrobial activities of Rumex crispus L. extracts. *Journal of agricultural and food chemistry*, *49*(8), 4083–4089.

16. Fontoura, R., Daroit, D. J., Correa, A. P. F., Meira, S. M. M., Mosquera, M., & Brandelli, A. (2014). Production of feather hydrolysates with antioxidant, angiotensin-I converting enzyme-and dipeptidyl peptidase-IV-inhibitory activities. *New biotechnology*, *31*(5), 506–513.

17. Hu, Y., Ge, C., Yuan, W., Zhu, R., Zhang, W., Du, L., & Xue, J. (2010). Characterization of fermented black soybean natto inoculated with Bacillus natto during fermentation. *Journal of the Science of Food and Agriculture*, *90*(7), 1194–1202.

18. Limón, R. I., Peñas, E., Torino, M. I., Martínez-Villaluenga, C., Dueñas, M., & Frias, J. (2015). Fermentation enhances the content of bioactive compounds in kidney bean extracts. *Food Chemistry*, *172*, 343–352.

19. Nasri, R., Chataigné, G., Bougatef, A., Chaâbouni, M. K., Dhulster, P., Nasri, M., & Nedjar-Arroume, N. (2013). Novel angiotensin I-converting enzyme inhibitory peptides from enzymatic hydrolysates of goby (Zosterisessor ophiocephalus) muscle proteins. *Journal of proteomics*, *91*, 444–452.

20. Salampessy, J., Reddy, N., Kailasapathy, K., & Phillips, M. (2015). Functional and potential therapeutic ACE-inhibitory peptides derived from bromelain hydrolysis of trevally proteins. *Journal of Functional Foods*, *14*, 716–725.

21. Corrêa, A. P. F., Daroit, D. J., Fontoura, R., Meira, S. M. M., Segalin, J., & Brandelli, A. (2014). Hydrolysates of sheep cheese whey as a source of bioactive peptides with antioxidant and angiotensin-converting enzyme inhibitory activities. *Peptides*, *61*, 48–55.

22. Balakrishnan, B., Prasad, B., Rai, A. K., Velappan, S. P., Subbanna, M. N., & Narayan, B. (2011). In vitro antioxidant and antibacterial properties of hydrolysed proteins of delimed tannery fleshings: comparison of acid hydrolysis and fermentation methods. *Biodegradation*, *22*(2), 287–295.

23. Nasri, R., Younes, I., Jridi, M., Trigui, M., Bougatef, A., Nedjar-Arroume, N., … Karra-Châabouni, M. (2013). ACE inhibitory and antioxidative activities of Goby (Zosterissessor ophiocephalus) fish protein hydrolysates: effect on meat lipid oxidation. *Food Research International*, *54*(1), 552–561.

24. He, R., Ju, X., Yuan, J., Wang, L., Girgih, A. T., & Aluko, R. E. (2012). Antioxidant activities of rapeseed peptides produced by solid state fermentation. *Food Research*

509 *International*, *49*(1), 432–438.

510 25. Corrêa, A. P. F., Daroit, D. J., Coelho, J., Meira, S. M. M., Lopes, F. C., Segalin, J., …
511 Brandelli, A. (2011). Antioxidant, antihypertensive and antimicrobial properties of
512 ovine milk caseinate hydrolyzed with a microbial protease. *Journal of the Science of*
513 *Food and Agriculture*, *91*(12), 2247–2254.

514 26. Ngo, D.-H., Kang, K.-H., Ryu, B., Vo, T.-S., Jung, W.-K., Byun, H.-G., & Kim, S.-K.
515 (2015). Angiotensin-I converting enzyme inhibitory peptides from antihypertensive
516 skate (Okamejei kenojei) skin gelatin hydrolysate in spontaneously hypertensive rats.
517 *Food Chemistry*, *174*, 37–43.

518 27. Shahidi, F., & Zhong, Y. (2008). Bioactive peptides. *Journal of AOAC International*,
519 *91*(4), 914–931.

520 28. Gupta, R., Beg, Q., Khan, S., & Chauhan, B. (2002). An overview on fermentation,
521 downstream processing and properties of microbial alkaline proteases. *Applied*
522 *microbiology and biotechnology*, *60*(4), 381–395.

523 29. Harwood, C. R. (1992). Bacillus subtilis and its relatives: molecular biological and
524 industrial workhorses. *Trends in biotechnology*, *10*, 247–256.

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

529 31. Zhang, S. B., Wang, Z., & Xu, S. Y. (2008). Antioxidant and antithrombotic activities
530 of rapeseed peptides. *Journal of the American Oil Chemists' Society*, *85*(6), 521–527.

531 32. Ruiz-Ruiz, J., Dávila-Ortíz, G., Chel-Guerrero, L., & Betancur-Ancona, D. (2013).
532 angiotensin i-converting enzyme inhibitory and antioxidant peptide fractions from
533 hard-to-cook bean enzymatic hydrolysates. *Journal of Food Biochemistry*, *37*(1), 26–
534 35.

535 33. Chalamaiah, M., Jyothirmayi, T., Bhaskarachary, K., Vajreswari, A., Hemalatha, R., &
536 Kumar, B. D. (2013). Chemical composition, molecular mass distribution and
537 antioxidant capacity of rohu (Labeo rohita) roe (egg) protein hydrolysates prepared by
538 gastrointestinal proteases. *Food Research International*, *52*(1), 221–229.

539 34. Cheung, I. W. Y., Cheung, L. K. Y., Tan, N. Y., & Li-Chan, E. C. Y. (2012). The role
540 of molecular size in antioxidant activity of peptide fractions from Pacific hake
541 (Merluccius productus) hydrolysates. *Food Chemistry*, *134*(3), 1297–1306.

542 35. Nasri, R., Jridi, M., Lassoued, I., Jemil, I., Salem, R. B. S.-B., Nasri, M., & Karra-
543 Châabouni, M. (2014). The Influence of the Extent of Enzymatic Hydrolysis on
544 Antioxidative Properties and ACE-Inhibitory Activities of Protein Hydrolysates from
545 Goby (Zosterisessor ophiocephalus) Muscle. *Applied biochemistry and biotechnology*,
546 *173*(5), 1121–1134.

547 36. Chen, N., Yang, H., Sun, Y., Niu, J., & Liu, S. (2012). Purification and identification
548 of antioxidant peptides from walnut (Juglans regia L.) protein hydrolysates. *Peptides*,
549 *38*(2), 344–349.

37. Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., … Xue, S. J. (2008). Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chemistry*, *108*(2), 727–736.

38. Kim, S.-Y., Je, J.-Y., & Kim, S.-K. (2007). Purification and characterization of antioxidant peptide from hoki (Johnius belengerii) frame protein by gastrointestinal digestion. *The Journal of Nutritional Biochemistry*, *18*(1), 31–38.

39. Je, J.-Y., Park, P.-J., & Kim, S.-K. (2005). Antioxidant activity of a peptide isolated from Alaska pollack (Theragra chalcogramma) frame protein hydrolysate. *Food Research International*, *38*(1), 45–50.

40. Wu, H.-C., Chen, H.-M., & Shiau, C.-Y. (2003). Free amino acids and peptides as related to antioxidant properties in protein hydrolysates of mackerel (Scomber austriasicus). *Food research international*, *36*(9), 949–957.

Figures captions

Fig. 1. Antihypertensive capacity (as ACE-inhibitory activity) (a), DPPH radical scavenging activity (b) and reducing power (c) of tomato seed meal hydrolysate and control slurry at different concentrations.

Fig. 2. Size Exclusion Chromatography (SEC) profile of control slurry (a) and tomato seed meal hydrolysate (b) at a concentration of 26 mg/mL, in comparison to distribution of molecular weights of the standards on Sephadex G-50 column (c). Standard proteins used for column calibration include bovine serum albumin (66 kDa), Chymotrypsinogen (25.6 kDa), cytochrome C (13 kDa), Aprotinin (6.51 kDa), Bacitracin (1.42 kDa), and Tyrosine (0.18 kDa).

Fig. 3. ACE inhibitory activity (a), DPPH radical scavenging activity (b) and reducing power of different fractions obtained from size exclusion chromatographic fractionation of tomato seed meal hydrolysate and the control slurry.

Fig 4. MALDI-TOF LC-MS spectra of active fractions from tomato seed meal hydrolysate (——) and control slurry (– – –) after size exclusion chromatography fractionations; *m/z* from 150 to 850 Da. H and C in the figure correspond to hydrolysate and control, respectively.

Fig. 5. MALDI-TOF LC-MS spectra of active fractions from tomato seed meal hydrolysate (——) and control slurry (– – –) after size exclusion chromatography fractionations; *m/z* from 850 to 3000 Da. H and C in the figure correspond to hydrolysate and control, respectively.

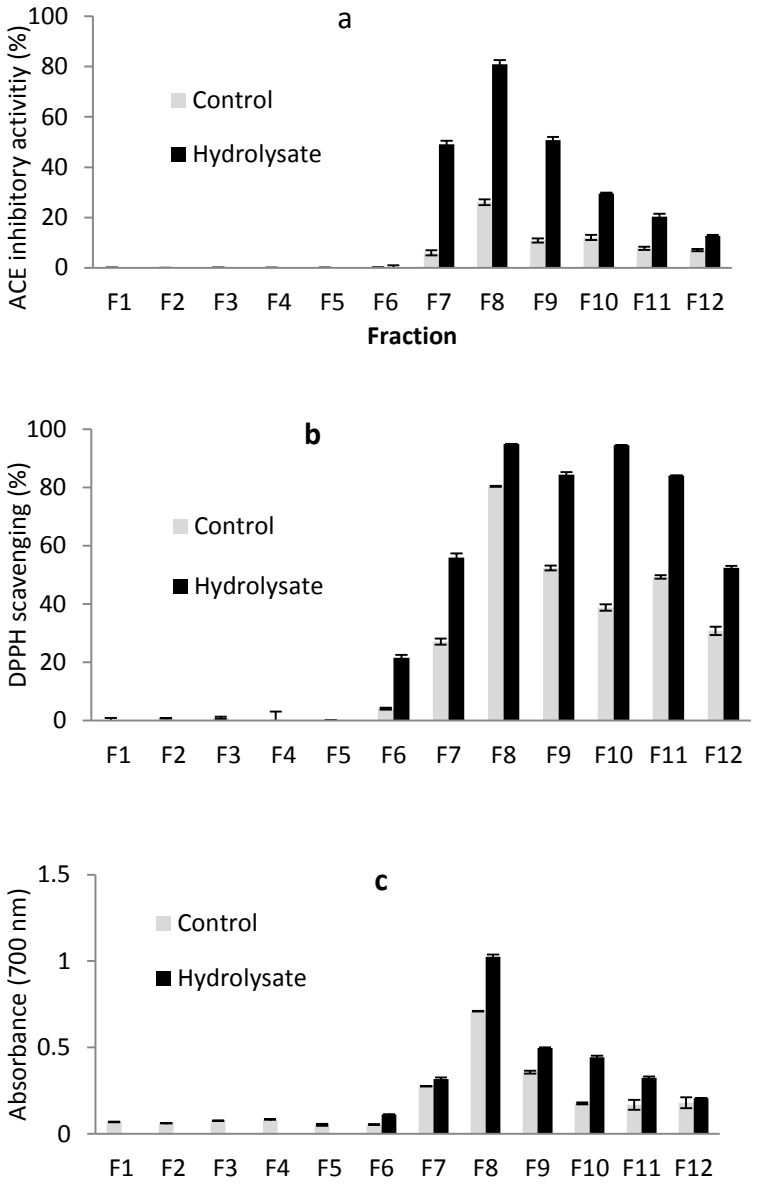

Fig. 3. ACE inhibitory activity (a), DPPH radical scavenging activity (b) and reducing power of different fractions obtained from size exclusion chromatographic fractionation of tomato seed meal hydrolysate and the control slurry.

Figure 4

Click here to download Figure Figure 4.docx

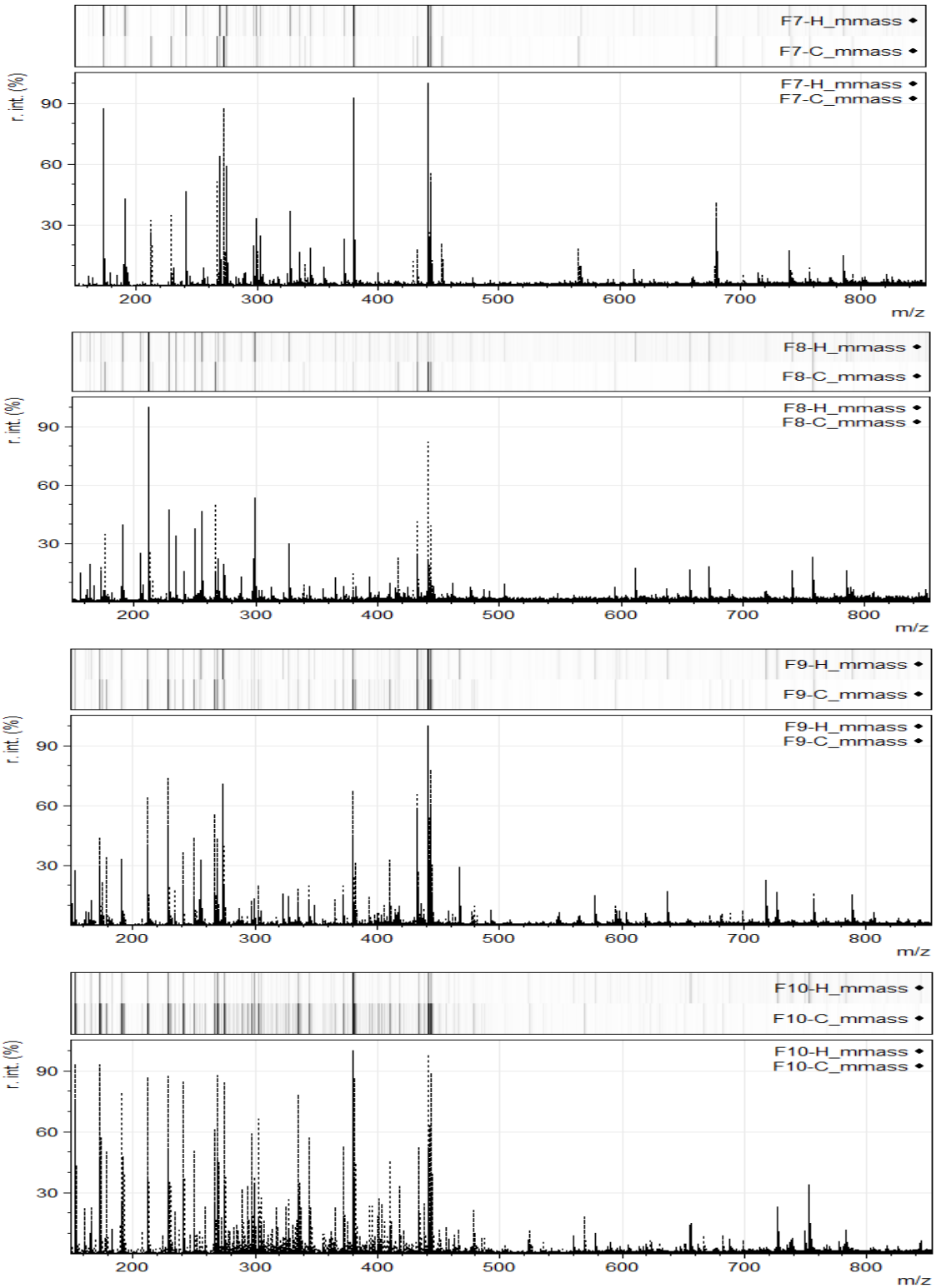

Fig 4. MALDI-TOF LC-MS spectra of active fractions from tomato seed meal hydrolysate (———) and control slurry (– – –) after size exclusion chromatography fractionations; *m/z* from 150 to 850 Da. H and C in the figure correspond to hydrolysate and control, respectively.

Figure 5

Click here to download Figure Figure 5.docx

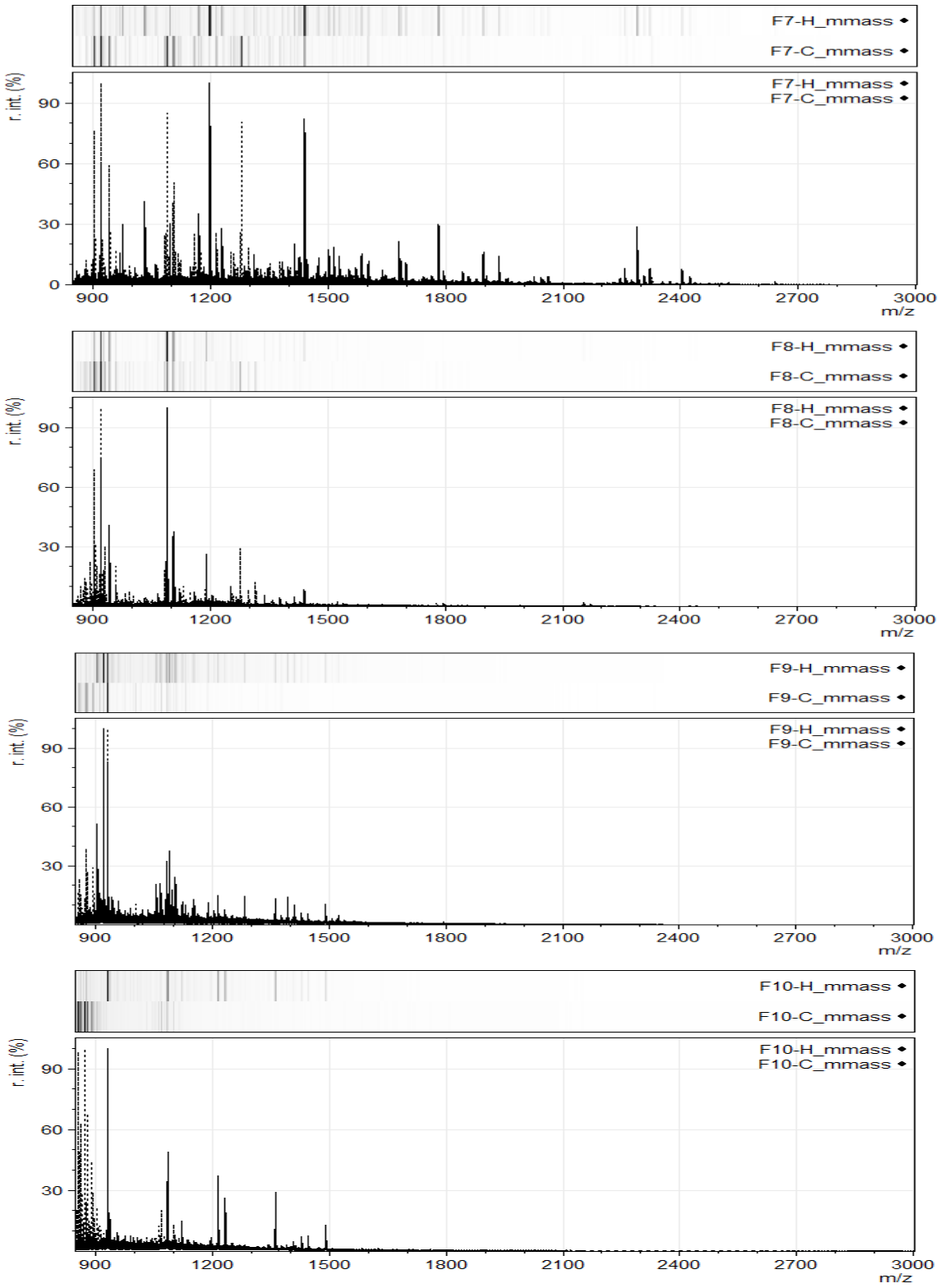

Fig. 5. MALDI-TOF LC-MS spectra of active fractions from tomato seed meal hydrolysate (——) and control slurry (– – –) after size exclusion chromatography fractionations; *m/z* from 850 to 3000 Da. H and C in the figure correspond to hydrolysate and control, respectively.

Table 1. Amino acid compositions of tomato seed meal hydrolysate (TSMH), and control slurry.

Amino acid	TSMH	Control
Asx	10.22[a] ± 0.10	9.49[b] ± 0.05
Glx	24.11[a] ± 0.11	22.80[a] ± 0.00
Ser	5.09[a] ± 0.09	5.03[a] ± 0.08
Gly	5.09[a] ± 0.04	4.88[b] ± 0.07
His	1.60[a] ± 0.02	1.68[a] ± 0.08
Thr	4.07[b] ± 0.07	4.24[a] ± 0.03
Ala	3.86[b] ± 0.11	4.32[a] ± 0.09
Arg	5.26[a] ± 0.15	4.34[b] ± 0.03
Pro	1.55[b] ± 0.37	4.98[a] ± 0.21
Tyr	3.97[a] ± 0.01	1.67[b] ± 0.02
Val	7.88[b] ± 0.05	11.73[a] ± 0.31
Met	1.50[b] ± 0.04	2.55[a] ± 0.04
Ile	3.46[b] ± 0.05	5.05[a] ± 0.11
Leu	6.14[b] ± 0.15	8.39[a] ± 0.38
Phe	7.30[a] ± 0.09	2.82[b] ± 0.01
Lys	8.89[a] ± 0.58	6.15[b] ± 0.05
Total	100.00	100.00
HAA	35.67[b]	41.51[a]
AAA	11.27[a]	4.49[b]
PCAA	15.75[a]	12.17[b]
NCAA	34.33[a]	32.29[b]
EAA	44.81[a]	44.28[a]

The values are percentages relative to total amino acid content (mg/mg) (Mean ± SD). Tryptophan and cysteine were destroyed during the acid digestion. Asx, aspartic acid and asparagine; Glx, glutamic acid and glutamine; combined total of hydrophobic amino acids (HAA) = valine, leucine, isoleucine, alanine, phenylalanine, tyrosine, methionine, and proline; total of aromatic amino acids (AAA) = phenylalanine, tyrosine; total of positively charged amino acids (PCAA) = arginine, histidine, lysine; total of negatively charged amino acids (NCAA) = Glx and Asx. In each row, the values with the same letter are not significantly different.