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

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

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11 [1]Department of Food Science and Technology, Gorgan University of Agricultural Sciences and Natural Resources,
12 Gorgan, Iran
13 [2]Instituto de Agroquimica y Technologia de Alimentos (CSIC), Avenue Agustin Escardino, Paterna, Valencia,
14 Spain
15 [3]Department of Microbial Biotechnology and Biosafety, Agricultural Biotechnology Research Institute of Iran
16 (ABRII), AREEO, P.O. Box 3135933151, Karaj, Iran
17 [4]Department of Food Science, Engineering and Technology, University of Tehran, Karaj, Iran

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28 *Corresponding author address: Fidel Toldrá (ftoldra@iata.csic.es) Tel: +34963900022 ext.
29 2112; Fax: +34963636301

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33 **Abstract**

34 The effects of amino acid composition and peptide molecular mass on ACE-inhibitory and
35 antioxidant activities of protein fragments obtained from tomato waste fermented using
36 *Bacillus subtilis* were evaluated. The addition of *B. subtilis* increased the relative amounts of
37 aromatic and positively-charged amino acids which have been described to influence the
38 biological activities of peptide fragments. IC_{50} values of hydrolysates for ACE-inhibitory and
39 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) scavenging activities were found to be 1.5 mg/mL
40 and 8.2 mg/mL, respectively. Size-exclusion chromatography (SEC) pattern of the
41 hydrolysate indicated the breakdown of parent proteins to smaller peptides with molecular
42 weights mainly below 1400 Da. MALDI-TOF mass spectrometry analysis revealed that the
43 highest ACE-inhibitory activity was due to peptides showing molecular mass range 500 - 800
44 Da, while the most active antioxidant peptides were found to be mainly at the two different
45 peptide weight ranges 500 – 800 Da and 1200 – 1500 Da.

46 *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].

71 Protein hydrolysates and peptides showing antioxidant and ACE inhibitory activities have
72 been described to be generated from different sources of plant and animal proteins. More
73 recently, food processing wastes and by-products have been of great interest to be considered
74 for the production of antioxidant and ACE-inhibitory peptides [5, 9, 10]. In this sense, the
75 tomato processing industry generates 7-7.5% of solid wastes. A total of 70-72 % of tomato
76 waste is pomace containing around 44% seeds (based on dried pomace). Protein constitutes
77 28% of tomato seed weight which is rich in globulin (60-70 %) [11]. So it is expected that a
78 considerable amount of high protein by-products could be generated from the tomato
79 processing industries all over the world. However, there are limited studies investigating the
80 potential of tomato waste proteins to produce added-value products.

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

88 **MATERIAL AND METHODS**

89 **Reagents and Chemicals**

90 2, 2′-diphenyl-1-picrylhydrazyl (DPPH) and ACE enzyme (from rabbit lung) were obtained
91 from Sigma Chemical Co. (St. Louis, MO, USA). Abz-Gly-p-nitro-phe-pro-OH
92 trifluoroacetate salt used as substrate was purchased from Bachem AG. (Bubendorf,
93 Switzerland). Microbial culture media were purchased from Sharlau (Barcelona, Spain).

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

96 **Tomato seed meal preparation**

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

102 **Bacterial culture**

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

110 **Fermentation and hydrolysis**

111 In order to obtain TSM hydrolysates, a submerged fermentation system with 250 mL
112 Erlenmeyer flasks (working volume 25 mL) containing TSM as substrate was applied as
113 previously described by Moayedi *et al*. [12]. The fermentation medium was comprised of
114 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,
115 the medium was inoculated (2%, v/v) with freshly prepared bacterial cells (1×10^8 CFU/mL)
116 and incubated for 24 h at 37 °C under agitation conditions. To stop the fermentation process

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

122 **Molecular mass distribution**

123 Size exclusion chromatography (SEC) system was used to estimate the molecular mass
124 distribution of protein and peptide fragments in TSMH and control slurry. For this purpose,
125 lyophilized TSMH and control slurry were completely dissolved in bi-distilled water (25
126 mg/mL), the solution was centrifuged at 10000×g for 10 min and the supernatant was filtered
127 through 0.22 µm syringe filter. Five mL of final clear solution was loaded onto a Sephadex
128 G-50 column. HCl 0.01 N at a flow rate of 15 mL/h was used as the eluent. Fractions of 5 mL
129 were collected at 20 min intervals by using an automated collector and the absorbance was
130 measured at 214, 254 and 280 nm using an UV-visible spectrophotometer (Agilent 8453 UV
131 spectrophotometer, Agilent Tech., Palo Alto, CA, USA). The column was calibrated by
132 loading standard gel filtration molecular weight markers, bovine serum albumin (66,000 Da),
133 Chymotrypsinogen (25,600 Da), Cytochrome C (13,000), Aprotinin (6,511.44 Da), Bacitracin
134 (1,422.69 Da), and Tyrosine (180 Da).

135 In order to evaluate the molecular weight distribution of peptide fragments and relate it to the
136 ACE-inhibitory and antioxidant activity, groups of fractions from an elution volume of 100
137 mL were pooled together (50 mL each), lyophilized and stored. New fractions were named
138 F1, F2, F3, F4, F5, F6, F7, F8, F9, F10, F11, and F12, corresponding to elution volumes of
139 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 400- 450, 450-500, 500-550, 550-
140 600, 600-650, and 650-700 mL, respectively.

141 **Amino acid analysis**

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

150 **MALDI-TOF mass spectrometry analysis**

151 The analysis was done in a 5800 MALDI-ToF/ToF instrument (ABSciex, CA, USA) in
152 positive reflectron mode (3000 shots every position) in two different ranges: 150-850 Da and
153 850-3500 Da; the laser intensity was manually adjusted to maximize the S/N ratio. Plate
154 model and acquisition method were calibrated by ABSCIEX calibration mixture (des-Arg1-
155 Bradykinin at 1fmol/µL; Angiotensin I at 2 fmol/µL; Glu1-Fibrinopeptide B at 1.3 fmol/µL;
156 ACTH (1–17 clip) at 2 fmol/µL; ACTH (18–39 clip) at 5 fmol/µL; and ACTH (7–38 clip) at
157 3 fmol/µL) in 13 positions. Dried hydrolysates were dissolved in 5% ACN; 0.1% TFA, and 1
158 µL of every sample was directly spotted on 10 positions in the MALDI plate and allowed to
159 air dry. Once dried, 0.5 µL of matrix solution (5 mg/mL of α-Cyano-4-hydroxycinnamic acid
160 (CHCA) in 0.1% TFA-ACN/H_2O (7:3, v/v) was spotted. The data was analyzed by using
161 mMass software (http://www.mmass.org/).

162 **Determination of ACE inhibitory activities**

163 The ACE inhibitory activities of TSMH and control were measured according to the
164 fluorescence-based method previously described by Sentandreu and Toldrá [2]. In this assay,
165 the internally quenched fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-
166 L-proline (Abz-Gly-Phe-(NO2)-Pro) is hydrolyzed by ACE to release the fluorescent product
167 o-aminobenzoylglycine (Abz-Gly). 50 µL of 3 mU/mL ACE preparation in Tris-base buffer
168 (150 mM, pH 8.3) was added to 50µL of sample, and the mixture was pre-incubated at 37 °C
169 for 10 min. Then, 200 µL of 150 mM Tris–HCl buffer (pH 8.3) containing 1.125 M NaCl and
170 10 mM Abz-Gly-Phe-(NO2)-Pro was added and the reaction mixture was incubated for 60
171 min at 37 °C. The fluorescence intensity was measured using excitation and emission
172 wavelengths of 355 and 405 nm, respectively. Bidistilled water was used as negative control
173 whereas captopril (0.1mg/mL) was used as positive control in the assay. ACE inhibitory
174 activity was expressed as percentage and measurements were done in triplicate (n=3). IC_{50}
175 value as the amount of peptide or hydrolysate required for inhibiting 50% of ACE activity
176 was also calculated.

177 **Determination of antioxidant activities**

178 **DPPH scavenging activity**

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

185 $$\%\text{DPPH scavenging activity} = (1 - \frac{As}{Ac}) \times 100$$

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

188 **Reducing power capacity**

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

198 **Statistical analysis**

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

202 **Results and Discussion**

203 **ACE-inhibitory activity of TSMH**

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

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

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

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

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

235 **Antioxidant capacities of TSMH**

236 DPPH radical scavenging activity of TSMH showed also a dose-dependent effect that
237 increases with the concentration (Fig. 1b), reaching a value of 57% at 10 mg/mL. The trend
238 of DPPH scavenging activity of TSMH was in agreement with previously reported studies in
239 which this activity was also dose-dependent [22, 23]. The IC_{50} value for TSMH sample was
240 8.2 mg/mL in DPPH scavenging activity. This value was higher than the IC_{50} value reported
241 by He *et al*. [24] in fermented rape seed using *B. subtilis* (165 µg/mL), but close to the IC_{50}
242 value observed by Jemil *et al*. [14] in fermented sardine protein using *B. subtilis* A26 (6
243 mg/mL approximately).

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

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

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

259 **Amino acid composition**

260 In order to know which is the contribution of *B. subtilis* A14h to the amino acids profile the
261 comparison between the amino acid relative composition of control and TSMH sample was
262 done, and the result is shown in Table 1. The relative composition of amino acids is different
263 between samples, but Glx (glutamic acid + glutamine) were found to be the most abundant
264 amino acids in both. The *B. subtilis* addition significantly increased relative amount of
265 tyrosine, phenylalanine and lysine in TSMH in comparison to the control (Table 1). In fact, a
266 2-fold higher percentage of aromatic amino acids in TSMH (11.27%) in comparison to
267 control slurry (4.49%) was observed. In particular, phenylalanine percentage showed a
268 marked increase from 3.51% in control to 7.30% in hydrolysate (TSMH). In addition,
269 arginine and lysine percentages increased in the hydrolysate which resulted in a higher
270 amount of total positively charged amino acids from 12.17% (in control) to 15.75% (in
271 TSMH).

272 Antioxidant and ACE inhibitory activities of peptides and protein hydrolysates are influenced
273 by factors like protein source, hydrolysis conditions, degree of hydrolysis, molecular mass,
274 and amino acid composition as well as the position of amino acids in the peptide sequences.
275 In this sense, the correlation between specific amino acid groups and biological activities has
276 been described in different studies about pea and apricot almond protein hydrolysates [23,
277 26].

278 It has been shown that hydrophobic amino acid residues like leucine, valine, alanine,
279 tyrosine, phenylalanine, or tryptophan, can act as competitive ACE-inhibitors as they
280 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

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

307 **Molecular mass distribution after Size-Exclusion Chromatography (SEC)**

308 As it has been previously described, the antioxidant and ACE inhibitory activity is closely
309 related to the molecular mass of peptides. Bioactive peptides with lower molecular mass may
310 have higher chance to cross the intestinal barrier and exert the biological effects. In fact, main
311 antioxidant and ACE-inhibitory peptides described usually contain 2-20 amino acid residues
312 per molecule. Thus, in order to better understand the role of molecular mass distribution
313 affecting antioxidant and antihypertensive capacities of TSMH and to evaluate the changes of
314 different protein fractions during fermentation, TSMH and control slurry were individually
315 fractionated using SEC. SEC profiles of both TSMH and control slurry using a Sephadex G-
316 50 column are presented in Fig. 2. Control sample showed six main peaks with the major
317 peak in the molecular range of 6500 – 13000 Da (fractions from 35 to 60) according to the
318 analysed standards (see Fig 2.c). The first peak includes proteins having a molecular mass of
319 13000 – 66000 Da. SEC profile also showed an important peak corresponding to low
320 molecular weight fragments since they are eluting later than the bacitracin standard (1423
321 Da). Regarding TSMH sample, SEC profile was different from control showing two main
322 peaks eluting later than the bacitracin standard (1423 Da). Comparing both SEC patterns
323 showed in Fig. 2, it could be concluded that hydrolytic activities of proteases produced by *B.*
324 *subtilis* during fermentation efficiently enhanced the fragmentation of high molecular mass
325 proteins low molecular mass peptides mainly less than 1423 Da. TSMH peak corresponding
326 to fractions between number 116 and 135 mainly contains free amino acids. In this sense,
327 different *B. subtilis* strains have shown to produce a mixture of endo-peptidases and
328 exopeptidases [29].

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

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

345 Regarding the effect of peptide size on antioxidant activity, different results were observed
346 depending on the type of antioxidant analysis. DPPH scavenging assay showed the highest
347 antioxidant percentage in fractions F8 and F10 with similar results, whereas the peak value
348 for ferric reducing power assay was observed only on F8. In all cases, TSMH indicated
349 higher antioxidant activity than did control. In this sense, some researchers reported that
350 molecular mass below 3000 Da was the most effective size [37], whereas others suggested
351 1000 to 3000 Da [38], and even below 1500 Da as the most active sequence sizes [39]. Wu *et*
352 *al.* [40] observed that mackerel hydrolysate fractions containing peptides with approximately
353 1400 Da displayed higher protective effect against lipid oxidation in comparison to fractions

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

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

365 **MALDI-TOF mass spectrometry analysis**

366 The results of MALDI-TOF analysis of different active fractions from SEC fractionation of
367 TSMH and control slurry are shown in Fig. 4 and Fig. 5 including *m/z* ranges of 150-850 and
368 850-3000, respectively. In this respect, fractions F7, F8, F9 and F10 in SEC profile that
369 showed the highest ACE and antioxidant activities correspond to F7-H, F8-H, F9-H and F10-
370 H (for TSMH) and F7-C, FF8-C, F9-C and F10-C (for control slurry), respectively. This
371 method has been widely applied to confirm the extent of hydrolysis and to determine
372 molecular weight distribution in protein hydrolysates [8, 26]. In fact, fractions F10-H and
373 F10-C (the late fractions in SEC profile) showed a higher amount and ion intensity of
374 peptides with molecular weights below 500 Da than those in other groups. In contrast, the
375 number of peptides in the range from 900 to 2700 Da was found to be higher in F7-H.
376 Moreover, MALDI-TOF spectra confirmed higher amount of peptides in TSMH fractions
377 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.

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COMPLIANCE WITH ETHICAL STANDARDS

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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.

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565 **Figures captions**

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

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

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

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

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

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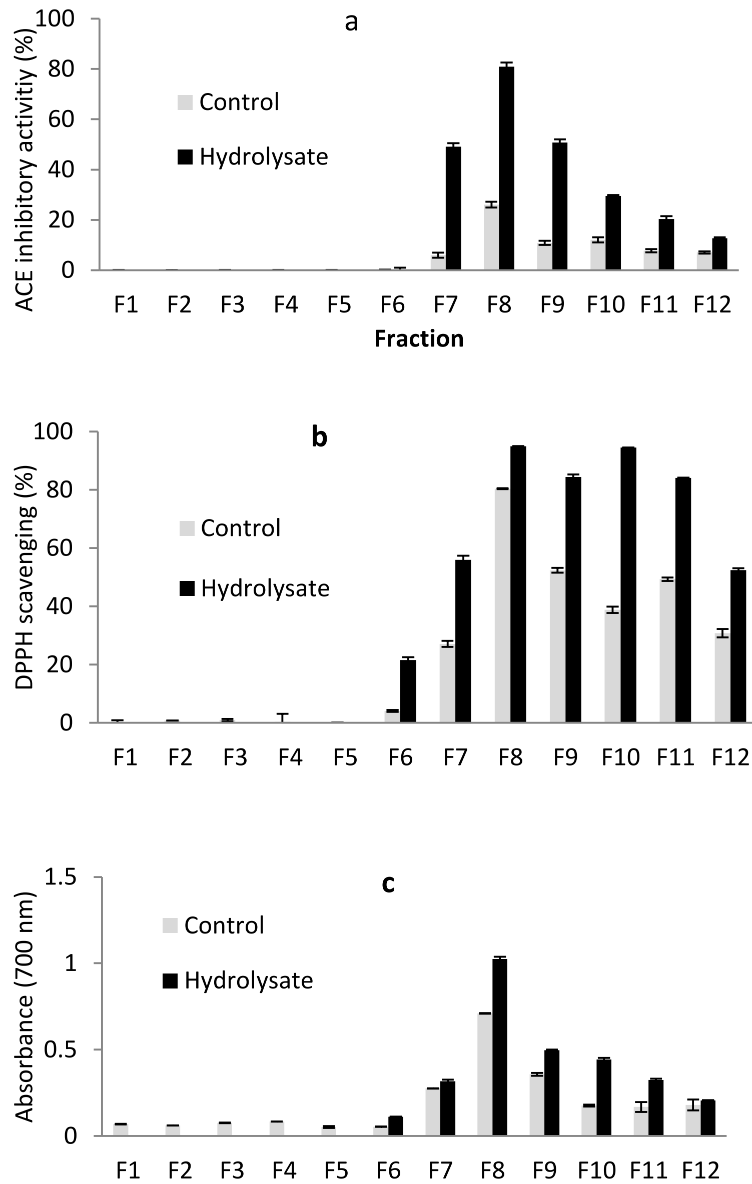

Fig. 3. ACE inhibitory activity (a), DPPH radical scavenging activity (b) and reducing power of different fractions obtained from size exclusion chromatographic fractionation of tomato seed meal hydrolysate and the control slurry.

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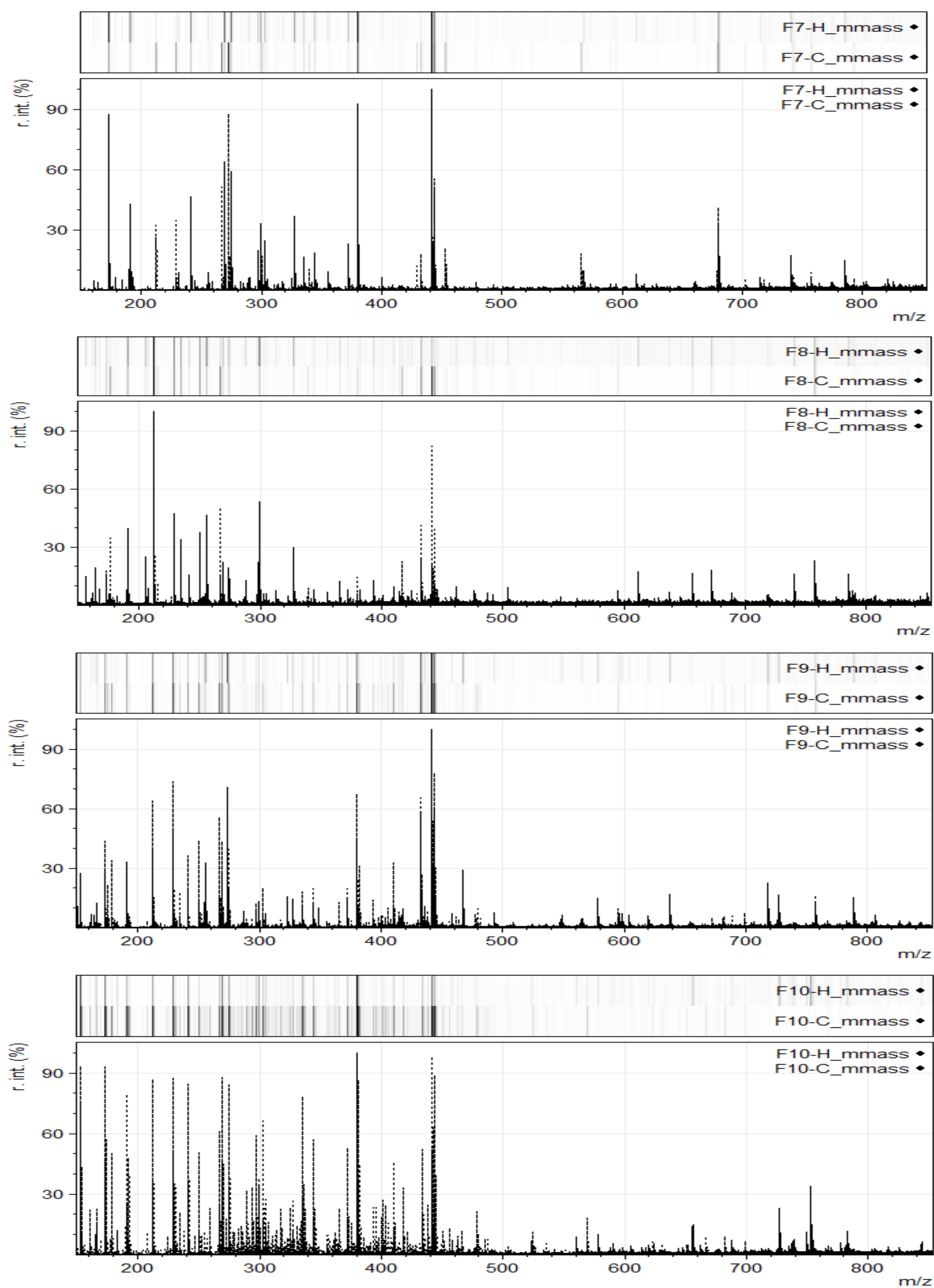

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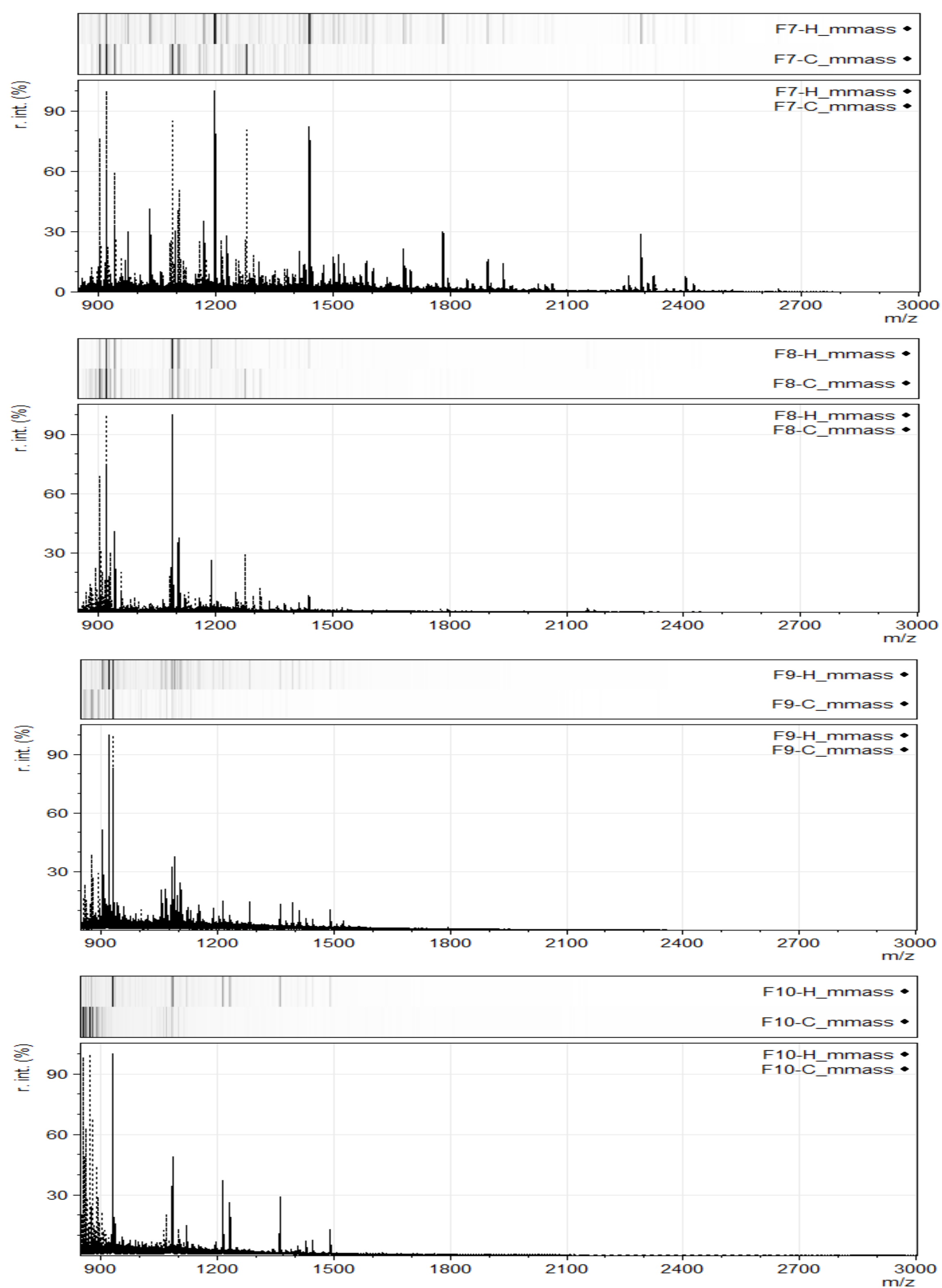

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Table 1. Amino acid compositions of tomato seed meal hydrolysate (TSMH), and control slurry.

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.