Stability of ACE inhibitory ham peptides against heat treatment and in

\textit{in vitro} digestion

Elizabeth Escudero, Leticia Mora, and Fidel Toldrá

\textit{Instituto de agroquímica y Tecnología de Alimentos (CSIC), Avd. Agustín Escandino, 7 46980, Paterna, Valencia, Spain}

Corresponding author: Tel: +343900022 ext.2112; fax: +343636301.

\textit{E-mail address:} ftoldra@iata.csic.es
Abstract

Angiotensin I-converting enzyme (ACE) inhibitory peptides derived from Spanish dry-cured ham have been examined for their stability during processing and after in vitro digestion. Peptides preserved almost the same ACE inhibitory activity after applying diverse heat treatments (from 50 to 117 °C), times of processing (from 3 to 60 min) and simulated in vitro digestion with gastrointestinal proteases. Peptides KAAAAP, AAPLAP, KPVAAP, IAGRP, and KAAAATP were the most potent peptides with IC$_{50}$ values ranging from 12.37 μM to 25.94 μM. Peptides IAGRP and PTPVP have also been identified in the processed sample (6 min at 117 °C), and in the in vitro digested sample. This study has shown the high stability of ACE inhibitory peptides derived from Spanish dry-cured ham against temperature of processing and gastrointestinal digestion as well as the powerful ACE inhibitory activity of some of the peptides identified in the Spanish dry-cured ham extract.

Keywords: Dry-cured ham, ACE inhibitory peptides, processing, gastrointestinal digestion, mass spectrometry.

1. Introduction

Many dietary proteins exert beneficial effects upon human health once released from their parent protein either by digestive enzymes during gastrointestinal digestion or by fermentation or ripening during food processing (Korhonen, Pihlanto-Leppala, Rantamaki & Tupasela, 1998). Bioactive peptides usually range in size from 2 to 50 amino acid residues and can exhibit different activities, such as antimicrobial, antioxidant, antithrombotic, antihypertensive, immunomodulatory, and opioid, among
others (Meisel & Fitzgerald, 2003; Lopez-Fandino, Otte & Van Camp, 2006; Toldrá and Reig, 2011). Peptides possessing specific biological properties make them potential ingredients of functional or health-promoting foods since they could reduce the risk of chronic diseases and promote human health (Hartmann & Meisel, 2007). The industrial application of these peptides and their incorporation into foods may affect the functional, nutritional, and biological properties of these peptides (Abdul-Hamid, Bakar & Bee, 2002; Vaslin, Le Guillou, Hannoucene & Saint Denis, 2006; Paul & Somkuti, 2009). On the other hand, when bioactive peptides are in the digestive tract after consumption, the activity of gastric enzymes may also affect their biological activities. In fact once ingested, proteins and peptides are subjected to hydrolysis by different enzymes such as pepsin, trypsin, or chymotrypsin. Some of the released peptides may exert a direct function at the gastrointestinal tract, whereas other peptides can be absorbed and reach target organs and tissues through systemic circulation (Shimizu, 2004). In previous studies, Escudero, Sentandreu and Toldrá (2010) demonstrated the generation of ACE inhibitory peptides after gastrointestinal digestion of pork meat. Moreover, in a recent study, Escudero, Toldrá, Sentandreu, Nishimura and Arihara (2012) investigated the in vivo antihypertensive activity of three novel peptides identified in the in vitro digest of pork meat, resulting in a significant decrease in systolic blood pressure after oral administration to spontaneously hypertensive rats. Our previous studies demonstrated that Spanish dry-cured ham is a natural source of peptides that show ACE inhibitory and in vivo antihypertensive activity in spontaneously hypertensive rats (Escudero, Aristoy, Nishimura, Arihara & Toldrá, 2012; Escudero, Mora, Fraser, Aristoy & Toldrá, 2013). Since there is a lack of information related to the influence of food processing and gastrointestinal digestion on the bioactivity of ACE inhibitory and antihypertensive peptides, the study of the
bioactive peptides stability during processing and after gastrointestinal digestion as well as the effects on their ACE inhibitory activity are of great importance. In this study, a Spanish dry-cured ham extract rich in bioactive peptides showing ACE inhibitory activity was used to study the impact of heat treatment and in vitro gastrointestinal digestion on its stability.

2. Materials and methods

2.1 Material and reagents

Dry-cured ham used in this study was a Designation of Origin of Teruel ham (D.O. Teruel, Spain), with a minimum time of ripening of fourteen months. The population used for the production of D.O. Teruel are maternal line Landrace and Large White crossbreds and paternal line purebred Duroc. Angiotensin-converting enzyme (from rabbit lung) was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A). Abz-Gly-p-nitro-phe-pro-OH trifluoroacetate salt was obtained from Bachem AG (Bubendorf, Switzerland). Pepsin (from hog stomach) was purchased from Fluka Chemie Gmbh (Buchs, Switzerland), and pancreatin (from porcine pancreas) was purchased from Sigma Chemical Co. (St. Louis, Mo., U.S.A). Other chemicals and reagents used were of analytical grade.

2.2 Peptide extraction

A sample of 100 g of Spanish dry-cured ham were minced and homogenized with 500 mL of 0.01N HCl in a Polytron® (Kinematica, Switzerland) for 5 min. The homogenate was kept at 4 ºC overnight for decanting, and the supernatant was filtered through a plastic mesh to retain the biggest pieces and then filtered again through a qualitative filter paper (Whatman^TM, UK). After that, the sample was freeze-dried and further submitted to solid phase extraction using an Oasis® HLB cartridge (35 cc, Waters, Ireland) in which the peptides were retained and then eluted using methanol-distilled
water (95:5, v/v). The eluted sample was lyophilized, constituting the starting material for the subsequent experiments.

2.3 Assay of ACE Inhibitory Activity

The ACE inhibitory activity of the Spanish dry-cured ham extract (control), processed sample, processed and further digested sample, and the synthesized peptides was measured according to the method developed by Sentandreu and Toldrá (2006). This assay is based on the ability of ACE to hydrolyze the internally quenched fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe-(NO₂)-Pro). A sample solution (50 μL dry-cured ham extract or synthesized peptide) was mixed with 50 μL of 150 mM Tris-base buffer (pH 8.3) containing 3 mU/mL of ACE, and the mixture was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of 200 μL of 150 mM Tris-base buffer (pH 8.3) containing 1.125 M NaCl and 10 mM Abz-Gly-Phe- (NO₂)-Pro, which was preincubated again for 10 min at 37 °C. Finally, the reaction mixture was then incubated for 45 min at 37 °C. The generation of fluorescence due to the release of o-aminobenzoylglycine (Abz-gly) by the action of ACE was measured using excitation and emission wavelengths of 355 and 405 nm, respectively. ACE inhibition of control, processed sample, and processed and subsequently digested sample is expressed as relative ACE inhibitory percentage and the ACE inhibition of synthesized peptides is expressed as IC₅₀. The IC₅₀ value is the concentration associated with 50% ACE inhibition in the reaction mixture. The experiments were performed by triplicate.

2.4 Stability of dry-cured ham ACE inhibitory peptides

Spanish dry-cured ham ACE inhibitory peptide solutions (5 mg/mL) were incubated at various temperatures, 50, 72, 90 and 117 °C for 6 min. On the other hand, the peptide solutions were also incubated at 117°C at different times 3, 6, 15, 30 and 60 min. All
solutions were taken to room temperature before the ACE inhibitory activity analysis, which was determined as described above. Triplicate assays were performed for each sample.

2.5 In vitro Digestion of dry-cured ham peptides

Stability of the peptides of Spanish dry-cured ham extract against in vitro gastric proteases was assessed using pepsin and pancreatin according to the method of Laparra, Vélez, Montoro, Barberá and Farré (2003) with some modifications. Pepsin solution in 6 M HCl (pH 2.0) was added to dry-cured ham extract at a 1:100 enzyme to substrate ratio. After 2 h of digestion at 37 ºC and continuous stirring, the enzyme was inactivated by adjusting the pH to 7.2 with 1 M NaHCO₃. Then, pancreatin was added at a 1:50 enzyme to substrate ratio. After 3 h of digestion at 37 ºC, enzyme activity was terminated by heating for 10 min at 95 ºC. The reaction mixture was freeze-dried and then reconstituted for ACE inhibitory activity determination.

2.6 Peptide identification by mass spectrometry in tandem (nESI-LC-MS/MS)

The nanoLC-MS/MS analysis was performed using an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA, USA) coupled to the quadrupole-time-of-flight (Q-ToF) TripleTOF® 5600+ system from AB Sciex Instruments (Framingham, MA, USA) that is equipped with a nanoelectrospray ionization source. Desalted dry-cured ham extracts were resuspended in H₂O with 0.1% of trifluoroacetic acid (TFA) to obtain a final concentration of 10 mg/mL. After centrifuge in cold for 3 min at 200xg, fifteen microlitres of each sample (control, processed dry-cured ham extract, and processed and digested dry-cured ham extract) were cleaned and concentrated using Zip-Tip C18 with standard bed format (Millipore Corporation,
Bedford, MA) according to manufacturer’s instructions. Five microlitres of the supernatant were injected into the LC-MS system through the autosampler. Samples were then preconcentrated on an Eksigent C18 trap column (3µ, 350µm x 0.5mm) (Eksigent of AB Sciex, CA, USA), at a flow rate of 3 µL/min and using 0.1% v/v TFA as mobile phase. After 5 min of preconcentration, the trap column was automatically switched in-line onto a nano-HPLC capillary column (3µm, 75µm x 12.3 cm, C18) (Nikkyo Technos Co, Ltd. Japan). The mobile phases consisted of solvent A, containing 0.1% v/v formic acid in water, and solvent B, containing 0.1% v/v FA in 100% acetonitrile. Chromatographic conditions were a linear gradient from 5% to 35% of solvent B over 90 min, and 10 min from 35% to 65% of solvent B, at a flow rate of 0.3 µL/min and running temperature of 30 ºC. The outlet of the capillary column was directly coupled to a nano-electrospray ionisation system (nano-ESI). The Q/ToF was operated in positive polarity and information-dependent acquisition mode, in which a 0.25-s ToF MS scan from m/z of 100 to 1200 was performed, followed by 0.05-s product ion scans from m/z of 100 to 1500 on the 50 most intense 1 to 5 charged ions.

2.7 Data analysis
Automated spectral processing, peak list generation, and database search were performed using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA) (http://www.matrixscience.com). The identification of protein origin of peptides was done using UniProt protein database, with a significance threshold $p<0.1$ and a FDR of 1.5%. The tolerance on the mass measurement was 0.3 Da in MS mode and 0.3 Da for MS/MS ions. 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).
2.8 Peptide Synthesis

Considering the low molecular mass and the structural requirements for ACE inhibition, some of the peptides identified from control sample, processed sample at 117 °C during 6 min, and processed and further *in vitro* digested sample, were synthesized by GenScript Corporation (Piscataway, NJ, USA) in order to assess their *in vitro* inhibition of ACE. The purity of the synthesized peptides was certified by analytical LC-MS.

3. Results and discussion

3.1 ACE inhibitory activity of Spanish dry-cured ham extract

Bioactive peptides can be found in intact food molecule but they are generally inactive within the sequence of protein molecule. During the processing of Spanish dry-cured ham an intense proteolysis takes place resulting in an accumulation of peptides of different sizes (mainly small peptides) (Mora, Sentandreu, Koistinen, Fraser, Toldrá & Bramley, 2009; Mora, Sentandreu, Fraser, Toldrá & Bramley, 2009) and free amino acids at the end of dry-curing (Toldrá, Aristoy & Flores, 2000). In previous studies it was found that peptide fractions from dry-cured ham exhibited relevant ACE inhibitory activity and *in vivo* antihypertensive activity (Escudero et al., 2012; Escudero et al., 2013).

Spanish dry-cured ham extract (control sample) was analyzed at different concentrations in order to get information about ACE inhibitory activity exerted (Fig.1) and select the most convenient concentration for the stability assays. As can be observed in figure 1, the extract showed inhibitory activity against ACE at all the assayed concentrations having a maximum activity of 60.7% at 10 mg/mL. For subsequent experiments the concentration of 5 mg/mL was chosen due to its proximity to the maximum ACE inhibitory activity and thus better elucidate the effect of temperature, time, and *in vitro* digestion on the bioactivity of the peptidic extract.
3.2 Impact of temperature and time on the activity of ACE inhibitory peptides

The bioactive peptides derived from Spanish dry-cured ham extract can be directly ingested from dry-cured ham, either raw or heat treated, or incorporated into other meat processed products. In this case, it is necessary to consider the stability of the ACE inhibitory activity among the typical temperature and time processing conditions. The temperature changes of the extracts were conducted by heating at 50, 72, 90 and 117 °C for 6 min (Fig. 2a) and the effect of time was assayed at 117 °C during 3, 6, 15, 30 and 60 min (Fig. 2b). These temperatures and times are commonly used in the food industry when processing meat products such as spreadable meat or ham-derived products. As shown in Fig. 2a and 2b, these peptides retained ACE inhibitory activity after applying different combinations of temperature and time of processing, which indicates that dry-cured ham ACE inhibitory peptides have good stability against heating. These results are consistent with those found in ACE inhibitory peptides derived from tuna cooking juice which exhibited good resistance to different temperatures (20-100°C for 2 h) reserving almost the same composition before and after treatments (Hwang, 2010). Furthermore, peptides derived from soy-protein were also stable after incubating at different temperatures (20-100°C for 2h) (Wu & Ding, 2002).

3.3 Effect of gastric enzymes on ACE inhibitory activity of dry-cured ham peptides

Digestion is one of the most important processes to release bioactive peptides which are inactive within the intact protein. Proteolytic enzymes can generate bioactive peptides during digestion (Hartman et al., 2007; Korhonen & Pihlanto, 2006; Escudero et al., 2010; Escudero, Sentandreu, Arihara & Toldrá, 2010). ACE inhibitory peptides could exert in vivo antihypertensive effect if they reach the cardiovascular system in an active form (Vermeirsssen, Van der Bent, Van Camp, Van Amerongen & Verstraete, 2004). So, after oral administration, peptides need to resist complete degradation by
gastrointestinal proteases and brush border peptidases, and they have to be absorbed through the intestinal wall with preservation of their biological activity. During this process, peptides can be degraded, resulting in an activation or inactivation of their biological activity. The ACE inhibitory activity of processed sample (117 °C during 6 min) and further digested by gastric proteases showed little change after in vitro incubation (Table 1) suggesting that peptides present in processed Spanish dry-cured ham extract may be resistant to digestion in the gastrointestinal tract or they may be partially degraded into smaller peptides keeping the antihypertensive biological activity. Previous reports have shown that small peptides still presented ACE inhibitory activity after digestion (Hwang, 2010; Wu & Ding, 2002; Jang, Cheorun & Lee, 2007). These results indicated that orally administered ACE inhibitory peptides could either keep their sequence integrity in the stomach or break into new smaller bioactive peptides that could reach the blood stream.

3.4 Identification and synthesis of ACE inhibitory peptides by tandem mass spectrometry

The desalted dry-cured ham sample used as control, the processed sample (117 °C during 6 min), and the same processed and subsequently in vitro digested sample, were analysed by nanoESI-LC-MS/MS mass spectrometry for the identification of their peptides content. Fig. 3 shows the total ion chromatograms (TICs) obtained after nano-liquid chromatography in the mass spectrometry system for control sample, processed sample at 117 °C for 6 min, and processed and subsequently in vitro digested sample. In the control and processed sample it can be observed an intensity of peaks very similar and, as the time progresses, the intensity of peaks decreases. On the other hand, in the processed and further digested sample it can be observed, in general, a decrease in the intensity of the detected ions, possibly because of changes in the characteristics of
peptides after digestion. Biological activities of peptides are related to their amino acid composition, sequence, size, and configuration (Matsui & Matsumoto, 2006). Most food protein-derived peptides with ACE inhibitory activity have low molecular mass, generally ranging from dipeptides to pentapeptides with molecular masses between 150 and 800 Da (Zhang, Wang & Wu, 2009). In this respect, the synthesised peptides had molecular mass <600 Da and contained between 5 and 7 amino acids. Considering the low molecular mass and structural requirements for ACE inhibition, some of the identified peptides were synthesised and their IC$_{50}$ calculated. Table 2 shows the sequence of the identified and synthesised peptides, the observed and calculated masses together with the charge states, and the protein of origin. So, six and seven of the peptides chosen for synthesis derive from myosin and titin proteins, respectively, whereas only four come from other type of proteins. The abundance of peptides derived from these proteins is due to the abundance of myosin and titin in skeletal muscle, and proves the high level of hydrolysis occurred during the dry-cured processing (Mora, Sentandreu, Koistinen, Fraser, Toldrá, and Bramley, 2009) as well as confirms that these proteins constitute a good source of ACE inhibitory peptides (Escudero et al, 2010).

All peptides share sequence with previously identified protein fragments that have been described as ACE inhibitors as shown in Table 2. In previous studies it has been reported that binding to ACE was strongly influenced by the residues at the three positions closest to the C-terminal site, especially hydrophobic amino acids as proline (Rohrbach, Williams, Rolsad, 1981) which is the most favourable C-terminal amino acid for binding to ACE. Also, the amino acid alanine close to the C-terminal position might also positively influence the binding to ACE (Majumder & Wu, 2009). In fact, best ACE inhibitory results in this study have been obtained for peptides KAAAAP,
AAPLAP, KPVAAP, IAGRP, and KAAAATP, with IC\textsubscript{50} values of 19.79, 14.38, 12.37, 25.94, and 25.64 μM, respectively, with an alanine and proline residue close to C-terminal and in the last C-terminal position, respectively. The activities of these novel peptides were extraordinarily higher than those previously identified from pork meat hydrolysates (Arihara, Nakashima, Mukai, Ishikawa & Itoh, 2001; Katayama et al., 2008). Peptide IAGRP has been identified by MS/MS in the control, the sample processed during 6 min at 117 ºC, and in the processed extract after \textit{in vitro} digestion with pepsin and pancreatin enzymes, proving the stability of this antihypertensive peptide after processing and digestion. Also the identity of peptide PTPVP has been confirmed in the control, the processed sample, and in the sample after \textit{in vitro} digestion. This peptide has been previously identified and tested \textit{in vitro} for its ACE inhibitory activity after a simulated gastrointestinal digestion of pork meat, showing an IC\textsubscript{50} of 256.41 μM (see Table 2) (Escudero et al., 2010). More recently, the \textit{in vivo} antihypertensive activity of PTPVP has been tested in spontaneously hypertensive rats (SHRs), resulting in a decrease of the systolic blood pressure of 24.52 mmHg (p<0.01) and 25.66 mmHg (p<0.01) at 4 and 6 h after single oral administration, respectively (Escudero et al., 2012). On the other hand, the synthesised peptide KAAAATP has shown an IC\textsubscript{50} value of 25.64 μM in this study (see Table 2). The fragment AAATP was also previously identified in a Spanish dry-cured ham and gave an IC\textsubscript{50} value of 100 μM (Escudero et al., 2013). These results are in agreement with the obtained results in this study. The processing of dry-cured ham extract under extreme conditions of temperature and time as well as the \textit{in vitro} digestion with pepsin and pancreatin enzymes, could be the responsible for the degradation of some of the ACE inhibitory peptides identified in the control extract. Despite this situation, the ACE inhibitory activity remained constant.
after processing and digestion, probably due to the presence of the tripeptide fragments

described in Table 2, which have been previously proved to be good antihypertensive
peptides. This fact could not be confirmed because the conditions of mass spectrometry
and data analysis tools used in this study does not allow the identification of di- and
tripeptides as their small size makes them difficult to be fragmented in the analysers,
and their short sequence decrease the possibility to find a specific origin protein as the
possibilities to be present in different sequences of Sus scrofa proteome increase with
the shortness of the peptide.

4. Conclusion

According to this study, the bioactivity of ACE inhibitory peptides was not affected by
heat treatments and they still showed relevant ACE inhibitory activity after in vitro
digestion by gastrointestinal proteases. A total of 16 peptides identified in the control
sample were synthesised and their IC$_{50}$ calculated being KAAAAP, AAPLAP, KPVAAP,
IAGRP, and KAAAATP the most active peptides presenting IC$_{50}$ values ranging from
12.37 µM to 25.94 µM. Peptide IAGRP has also been identified in the processed
sample during 6 min at 117 ºC, and in the processed and further in vitro digested sample.
Also, peptide PTPVP that has been previously described by Escudero et al. (2010;
2012) was identified in the control, processed sample, and processed and digested
sample. These findings prove the stability of ACE inhibitory activity of peptides
submitted to intense processing conditions and after digestion. ACE inhibitory activity
keeps constant probably due to the presence of the identified antihypertensive peptides
as well as small fragments resulting from their degradation occurred after processing
and in vitro digestion.

The knowledge of interactions of bioactive peptides with other food components during
processing and the evaluation of efficacy of bioactive peptides in animal model and
human clinical studies *per se* and in food systems will be crucial to ensure activity and bioavailability of these bioactive peptides.

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**Literature cited**


LEGENDS FOR THE FIGURES

**Figure 1.** ACE inhibitory (ACEI) activity of Spanish dry-cured ham extract (control) at different concentrations.

**Figure 2.** Stability of Spanish dry-cured ham-derived ACE inhibitory peptides after: a) 6 min incubation at various temperatures, and b) 117°C incubation at different times. The relative ACE inhibitory percent was calculated as the ratio of ACE inhibitory activity between the control and treatments. Bars represent means ± SD.
Figure 3. Total ion chromatograms (TICs) obtained after nano-liquid chromatography in the mass spectrometry system for control sample, processed sample at 117 °C for 6 min, and processed and subsequently in vitro digested sample.
Lines were numbered 5 to 5 and are now numbered consecutively 1 by 1.
**Table 1.** Activity of the Spanish dry-cured ham extract following processing (117 °C during 6 min), and processing and further *in vitro* digestion by gastrointestinal proteases.

<table>
<thead>
<tr>
<th>Sample</th>
<th>ACE inhibition (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>45.9±1.115</td>
<td>2.42</td>
</tr>
<tr>
<td>Processed sample</td>
<td>40.42±0.54</td>
<td>1.33</td>
</tr>
<tr>
<td>Processed + digested sample</td>
<td>42.01±3.44</td>
<td>8.18</td>
</tr>
</tbody>
</table>

All values are mean±standard deviation for triplicate experiments.
Table 2. Peptides from the desalted dry-cured ham extract (control sample) identified by nanoESI-LC-MS/MS and synthesised to test the ACE inhibitory activity.

<table>
<thead>
<tr>
<th>Peptide number</th>
<th>P&lt;sub&gt;a&lt;/sub&gt;</th>
<th>Sequence</th>
<th>P&lt;sub&gt;b&lt;/sub&gt;</th>
<th>Observed&lt;sup&gt;c&lt;/sup&gt; (m/z)</th>
<th>Charge&lt;sup&gt;d&lt;/sup&gt; (H&lt;sup&gt;+&lt;/sup&gt;)</th>
<th>Calculated&lt;sup&gt;d&lt;/sup&gt; Mr</th>
<th>Protein of origin</th>
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<tr>
<td>1</td>
<td>A</td>
<td>PAPPK&lt;sup&gt;φ&lt;/sup&gt;</td>
<td>E</td>
<td>509.30</td>
<td>1</td>
<td>508.30</td>
<td>Myosin light chain 1/3</td>
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<tr>
<td>2</td>
<td>A</td>
<td>KAAAAP</td>
<td>A</td>
<td>528.27</td>
<td>1</td>
<td>527.31</td>
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<tr>
<td>3</td>
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<td>G</td>
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<td>AAPLAP</td>
<td>I</td>
<td>539.34</td>
<td>1</td>
<td>538.31</td>
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<td>6</td>
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<td>KPVAAP</td>
<td>V</td>
<td>582.29</td>
<td>1</td>
<td>581.35</td>
<td>Myosin-XV</td>
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<tr>
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<td>8</td>
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<td>KPGRP</td>
<td>D</td>
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<td>510.24</td>
<td>Titin</td>
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<td>IAGRP*</td>
<td>L</td>
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<tr>
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<td>512.33</td>
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<tr>
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<td>F</td>
<td>TGLKP</td>
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<td>1</td>
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<td>15</td>
<td>P</td>
<td>AAATPL&lt;sup&gt;¥&lt;/sup&gt;</td>
<td>A</td>
<td>272.22</td>
<td>2</td>
<td>542.31</td>
<td>Epithelial splicing regulatory protein 2</td>
</tr>
<tr>
<td>16</td>
<td>V</td>
<td>KAAAATP</td>
<td>F</td>
<td>629.30</td>
<td>1</td>
<td>628.35</td>
<td>PR domain zinc finger protein 2</td>
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<tr>
<td>17</td>
<td>K</td>
<td>PTPVP*</td>
<td>K</td>
<td>510.25</td>
<td>1</td>
<td>509.29</td>
<td>Titin</td>
</tr>
</tbody>
</table>

<sup>a</sup> Amino acid residue preceding the peptide sequence; <sup>b</sup> amino acid residue following the peptide sequence; <sup>c</sup> Relation of mass/charge (m/z) observed in the nanoLC-MS/MS system; <sup>d</sup> Calculated relative molecular mass in Daltons of the matched peptide; <sup>φ</sup> Peptide PAPPK has been also identified in the extract processed at 117°C during 5 min; <sup>*</sup> Peptides IAGRP and PTPVP have been identified in the processed extract at 117°C during 5 min and after digestion. PTPVP peptide was synthesized and tested in vitro and in vivo in a previous work as indicated in Table 2; <sup>¥</sup> Peptide AAATPL has been identified in the processed extract after digestion.
Table 3. ACE inhibitory activity (IC$_{50}$) of synthesised peptides and previously published ACE inhibitory fragments of these peptides.

<table>
<thead>
<tr>
<th>Peptide Number</th>
<th>Sequence</th>
<th>IC$_{50}$ (µM)</th>
<th>Previously identified ACE inhibitory sequences$^a$</th>
<th>Sequence</th>
<th>IC$_{50}$ (µM)</th>
<th>References$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>AMNPP</td>
<td>304.50</td>
<td>MNPP 945.5</td>
<td>AAP</td>
<td>n.d.</td>
<td>Meisel H. (1993)</td>
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<tr>
<td>14</td>
<td>TGLKLP</td>
<td>51.57</td>
<td>LKP 0.32</td>
<td></td>
<td></td>
<td>Fujita H., Yokoyama K. &amp; Yoshikawa M. (2000)</td>
</tr>
<tr>
<td>15</td>
<td>AAATPL</td>
<td>n.d.</td>
<td>AAATP 100</td>
<td></td>
<td></td>
<td>Escudero E., Mora, L., Fraser P.D., Aristoy M.C., Arhara K. &amp; Toledo F. (2013)</td>
</tr>
<tr>
<td>16</td>
<td>KAAAATP</td>
<td>25.64</td>
<td>AAATP 100</td>
<td></td>
<td></td>
<td>Escudero E., Mora, L., Fraser P.D., Aristoy M.C., Arhara K. &amp; Toledo F. (2013)</td>
</tr>
</tbody>
</table>

$^a$ ACE inhibitory sequences previously identified that share amino acid residues with the sequences identified in this work.; $^b$ IC$_{50}$ of the fragments previously published.; $^*$ PTPVP peptide was synthesized and tested in vitro and in vivo in a previous work.; n.d. means non-detected.
Figure 1
Figure 2

Figure 2
Figure 3

- Control
- Processed sample 6 min at 117 °C
- Processed and in vitro digested sample

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