Transepithelial transport of dry-cured ham peptides with ACE inhibitory activity through a Caco-2 cell monolayer

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Abstract

~~Angiotensin converting enzyme (ACE) inhibitory peptides are been extensively studied as an alternative to synthetic drugs for the treatment of hypertension. Recent studies have shown that dry-cured ham is an important source of naturally generated bioactive peptides, especially showing ACE inhibitory activity. However, due to their excessive degradation by digestive and brush-border enzymes, it is not clear whether these peptides resist intestinal absorption and reach the blood stream where they may exert their antihypertensive effect. So, d~~Dry-cured ham extracts and specific pure peptides naturally generated during the dry-curing process, showing ACE inhibitory activity, have been studied for their stability during transepithelial transport in a Caco-2 cell monolayer. The ACE inhibitory activity of transport samples was assayed, reaching the highest values in apical samples after 15 min ~~of~~ incubation. In basal solutions, the highest ACE inhibition was observed for peptides AAPLAP and KPVAAP after 60 min of cellular transport. However, when basal samples were four times concentrated, a considerable increased ACE inhibitory activity was observed ~~in these peptides from 15 min of incubation~~. Fragments generated by cellular activity were detected by using tandem mass spectrometry ~~MS~~techniques, showing that AAATP, AAPLAP, and KPVAAP were hydrolysed during the transport, although KPVAAP was also absorbed intact~~ly~~. This study highlights the potential of intact dry-cured ham peptides as well as their fragments to be absorbed across the intestinal epithelium and reach the blood stream to exert an antihypertensive action.

Keywords: Dry-cured ham, ACE inhibitory peptides, Caco-2 cell monolayer, intestinal transport, mass spectrometry.

1. Introduction

Angiotensin I converting enzyme (ACE) is a dipeptidyl carboxypeptidase which plays an essential role as a regulator of blood pressure. ACE converts angiotensin I to the potent vasoconstrictor angiotensin II, which also induces the release of aldosterone. Moreover, ACE inactivates bradykinin, which has vasodilator activity. As a result, the action of ACE on these two systems is responsible for hypertension, the most common type of cardiovascular disease (Skeggs, Kahn, & Shumway, 1956; Ondetti, Rubin, & Cushman, 1977; Unger, 2002). To exert effects on blood pressure, ACE-inhibitory compounds ~~such as antihypertensive peptides~~ need to resist the degradation by gastrointestinal proteases and brush border peptidases, be absorbed through the intestinal epithelium~~,~~ and finally reach the bloodstream in an active form (Vermeirssen, Augustijns, Morel, Van Camp, Opsomer, & Verstraete, 2005).

Spanish dry-cured ham has recently been investigated as a natural source of antihypertensive peptides, evaluating the ACE inhibitory activity of water soluble fractions of dry-cured ham extracts (Escudero, Aristoy, Nishimura, Arihara, & Toldrá, 2012), and identifying some of the peptides responsible for this inhibitory effect (Escudero, Mora, Fraser, Aristoy, Arihara, & Toldrá, 2013). Moreover, Escudero, Mora, and Toldrá (2014) have recently reported that the ACE inhibitory activity of dry-cured ham peptides persists after *in vitro* digestion with gastric proteases, which may be due to ~~both resistance~~ the stability of the existing antihypertensive peptides to digestion ~~as well as~~and the generation of small fragments ~~with~~ showing ACE inhibitory activity. Furthermore, the *in vivo* antihypertensive activity of dry-cured ham extracts and peptide AAATP has been studied, showing a decrease in systolic blood pressure after their oral administration to spontaneously hypertensive rats (SHR) (Escudero et al., 2012; Escudero et al., 2013).

Transport assays through Caco-2 cells, which is a cell line derived from human colon adenocarcinoma, have been established as a model for small intestinal transport of drugs and food compounds. Differentiated Caco-2 cells maintain the morphology and function of mature enterocytes and express brush border ~~proteases~~ peptidases and transporters ~~and peptidases~~ that may affect peptide stability and transport, being therefore utilised to predict the absorption in the small intestine (Hidalgo, Raub, & Borchardt, 1989; Yee, 1997). In this ~~sense~~regard, several studies have been focused on studying the transepithelial transport of antihypertensive peptides derived from different food products such as egg or milk in a qualitative way (Miguel, Dávalos, Manso, De la Peña, Lasunción, & López-Fandiño, 2004; Vermeirssen et al., 2005; Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008; Bejjani & Wu, 2013). However, to the best of our knowledge, there are no transport studies based on peptides showing ACE inhibitory activity derived from meat or meat products. In this work, the Caco-2 cell line was used to study the brush border degradation and transepithelial transport of ACE inhibitory peptides derived from dry-cured ham. In contrast with other studies focused on the ACE-inhibitory activity of food peptides, this study investigates the ACE-inhibitory effect of dry-cured ham peptides transported through the intestinal epithelium and hence show their potential to exert an antihypertensive action *in vivo*.

~~In addition, the ACE inhibitory activity of the transported peptides was measured to evaluate their final antihypertensive potential.~~

2. Material~~s~~ and methods

2.1 Material and reagents

Dulbeco's Modified Eagle's Medium (DMEM), GlutaMAX™, phosphate buffered saline (PBS), and nonessential amino acids were procured from Life Technologies

(Ghent, Belgium), whereas fetal bovine serum was from Greiner Bio-One (Vilvoorde, Belgium). Angiotensin-converting enzyme (from rabbit lung) was purchased from Sigma Chemical Co. (St. Louis, Mo., USA), and *o*-aminobenzoylglycyl-*p*-nitro-*L*-phenylalanyl-*L*-proline (Abz-Gly-p-nitro-Phe-Pro-OH) trifluoroacetate salt was from Bachem AG. (Bubendorf, Switzerland). Methanol HPLC grade was from Sharlab, S.L. (Barcelona, Spain). All other chemicals and reagents used were of analytical grade.

2.2 Dry-cured ham extracts and peptides

The study was done using extracts from different types of dry-cured ham. Samples M1 and S1 were obtained from Spanish dry-cured hams with ten months of processing, which were ~~submitted~~ subjected to extraction and deproteinisation according to the methodology described by Escudero et al. (2012). In the case of sample S1, the extract was fractionated by gel filtration chromatography using a Sephadex G-25 column (2.5 × 65 cm), and fractions corresponding to elution volumes from 200 to 320 mL were pooled together and lyophilised~~dried~~. These fractions were selected because they have shown the maximum ACE inhibitory activity, reaching values of 80% of inhibition in a previous study (Escudero et al., 2012). In addition, Designation of Origin of Teruel hams with a minimum time of ripening of fourteen months were used to obtain samples M2 and M3. Peptide extraction was done according to the method described by Escudero et al. (2014) for sample M2, while sample M3 was ~~submitted~~ subjected to extraction and deproteinisation following the same procedure as described above for sample M1 and S1. All dry-cured ham extracts were desalted by solid phase extraction using an Oasis® hydrophilic-lipophilic balance (HLB) cartridge (35 cc, Waters, Ireland), where peptides were retained and then eluted with 50% methanol. Finally, the eluates were lyophilised for the transport experiment across Caco-2 cells.

Since dry-cured ham extract samples contain a complex mixture of peptides that difficult a detailed study, three peptides (AAATP, AAPLAP, and KPVAAP) previously identified as ACE inhibitors in dry-cured ham (Escudero et al., 2013; Escudero et al., 2014) were selected. In fact, peptides AAPLAP and KPVAAP were chosen based on their potent ACE inhibition, with IC_{50} values of 14.38 and 12.37 µM, respectively. Whereas, AAATP (IC_{50} value of 100 µM) was selected for its good *in vivo* antihypertensive action in the SHR model (Escudero et al., 2013). These three peptides were synthetised by GenScript Corporation (Piscataway, NJ, USA) at the highest purity certified using liquid chromatography - mass spectrometry (LC-MS) ~~LC-MS~~ analysis for subsequent transport experiments.

2.2 Caco-2 cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in DMEM containing 4.5 g/L glucose, supplemented with GlutaMAX™, 10% fetal bovine serum and 1% nonessential amino acids, at 37 ~~°~~°C in a humidified atmosphere containing 10% CO_2. The passage number of the cells used in this study was between 30 and 35. For transport experiments, cells were seeded at a density of 20,000 cells/well on high throughput screening (HTS) Transwell®-24 well permeable supports (0.4 µm pore polyester membrane, 6.5 mm inserts, 0.33 cm^2 cell growth area; Costar, Corning, Birmingham, UK). The culture medium was replaced every 2-3 days and cells were allowed to differentiate for at least 21 days before experiments. Cell monolayer integrity was checked visually by phase-contrast microscopy, and the transepithelial electrical resistance (TEER) of Caco-2 cells of this batch assessed with an automated tissue resistance measurement system (REMS, World Precision Instruments, Hertfordshire, UK) was higher than 300 Ω*cm².

2.3 Transport studies

Differentiated Caco-2 cells were gently rinsed twice with PBS and then incubated with PBS for 1 day at 37 °C in 10% CO_2 prior to the transport assays. After removing the PBS from all wells, 1 mg/mL of the samples dissolved in PBS ~~were~~ was added to the apical chambers (200 µL), whereas fresh PBS was added to the basolateral chambers (1 mL). A control sample, containing only PBS and no peptides, was included in the experimental setup, and samples from apical and basolateral sides were taken before (0 min) and during incubation at different time points of 15, 30, and 60 min. The action of cell proteases on samples after the transport study was immediately stopped by adding 9 volumes of methanol, centrifuged at 7500*g* for 10 min, and finally the supernatant containing the peptides was taken and dried for the following analysis. All experiments were ~~conducted~~ done in triplicate.

2.4 ACE inhibitory activity

Apical and basal samples taken from the transepithelial transport at different times were analysed for ACE inhibitory activity. For this purpose, dried apical samples were dissolved to the original volume in bidistilled water, whereas dried basal samples were first dissolved in a volume four-fold smaller than the original one to determine the ACE inhibition in concentrated samples, and then ~~redissolved~~ diluted to the original volume to test again. Basal samples were concentrated in order to be comparable in terms of peptide concentration with apical samples. Additionally, the ACE inhibitory activity of several synthetised fragments derived from the degradation of AAATP, AAPLAP, and KPVAAP through the cellular transport was evaluated. ~~In all studied cases, t~~The ACE inhibitory activity was measured according to the method developed by Sentandreu and Toldrá (2006), which is based on the ability of ACE to hydrolyse the internally quenched fluorescent substrate ~~*o*-aminobenzoylglycyl-*p*-nitro-*L*-phenylalanyl-*L*-proline~~ ~~(~~Abz-Gly-Phe(NO_2)-Pro~~)~~. The assay was done in triplicate. ACE inhibition of samples

is ~~expressed~~ showed as ACE inhibitory percentage and ~~as~~ IC_{50} value, which is the peptide concentration that inhibits 50% of ACE activity in the reaction mixture.

2.5 MALDI-ToF/ToF mass spectrometry

Apical and basal samples taken after the transport of peptides AAATP, AAPLAP, and KPVAAP across Caco-2 cells were analysed using matrix-assisted laser desorption/ionisation time-of-flight/time-of-flight (MALDI-ToF/ToF) mass spectrometry to determine the molecular mass of the peptide mixture and detect those possible peptides resulting from the degradation of the precursor peptides. The analysis was done in MS mode, and a total of 1 µL of every sample was directly spotted on the MALDI plate and allowed to air dry. Then~~,~~ 0.5 µL of matrix solution (which contains 5 mg/mL of α-cyano-4-hydroxycinnamic acid (HCCA; Bruker Daltonics, Germany) in 0.1% ~~TFA-ACN/H_2O~~ trifluoroacetic acid - acetonitrile/water (7:3, v/v) was spotted. The analysis was done in a 5800 MALDI ToF/ToF instrument (AB Sciex, MA, USA) in positive reflectron mode (3000 shots every position) in the range from 150 to 800 ~~Da~~ *m/z*~~range~~; the laser intensity was manually adjusted to maximize the S/N ratio. The system was adjusted with voltages of 15 and 3 kV in the source and reflector detector, respectively. Previously, the plate model and the acquisition methods were calibrated by AB Sciex calibration mixture (1 fmol/µL des-Arg1-Bradykinin; 2 fmol/µL Angiotensin I; 1.3 fmol/µL Glu1-Fibrinopeptide B; 2 fmol/µL Adrenocorticotropic hormone (ACTH) (1–17 clip); 5 fmol/µL ACTH (18–39 clip); and 3 fmol/µL ACTH (7–38 clip)) in 13 positions.

The candidate precursors were then selected for every position in the MS/MS (ToF/ToF) analysis in order to confirm their presence. In this ~~sense~~regard, ten of the most intense precursors according to the threshold criteria minimum signal-to-noise: 10, minimum cluster area: 500, maximum precursor gap: 200 ppm, and maximum fraction

gap: 4, were selected. The MS/MS method was calibrated using Angiotensin I MS/MS spectra in 13 positions, and data was acquired using the default 1kV MS/MS method. Manual analysis of data was done using mMass – Open Source Mass Spectrometry Tool Software v.5.5 (http://www.mmass.org) (Strohalm, Kavan, Novák, Volný, & Havlíček, 2010). The search of peptide sequences previously identified showing ACE inhibitory activity was done using BIOPEP database (http://www.uwm.edu.pl/biochemia/index.php/en/biopep).

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2.6 Statistical analysis

Statistical analysis was done using Statgraphics Centurion XVI software. One-way analysis of variance (ANOVA) was performed and ~~the~~ mean and standard deviation were reported. Fisher's multiple range test was carried out to analyse significant differences among mean values at $p < 0.05$.

3. Results and discussion

3.1 Transepithelial transport of peptides across Caco-2 cell monolayer

In order to simplify the study of dry-cured ham extracts which contain a complex mixture of thousands of peptides, the apical to basal flux transport in Caco-2 cell monolayers was mainly studied with the purified peptides (AAATP, AAPLAP, and KPVAAP). For that, 1 mg/mL of every sample was added to the apical side~~,~~ and samples were taken from both apical and basal compartments at different times: 0, 15, 30, and 60 min.

The evolution of peptides AAATP, AAPLAP, and KPVAAP during their transcellular transport was then monitored by using MALDI-ToF mass spectrometry to determine the profile of peptides according to their molecular masses. As illustrated in Figure 1, there are more and smaller peptides in the samples taken from the basal sides than those of

the apical ones, suggesting a partial degradation of the precursor peptides into smaller fragments by the action of cell peptidases ~~as well as~~and their absorption through the Caco-2 cell monolayer. The size, amino acid sequence, charge, hydrogen-bonding capacity, non-enzymatic glycosylation, and hydrophobicity of peptides are critical in determining their susceptibility to peptidases and their permeability across the intestinal epithelium (Pauletti, Okumu, & Borchardt, 1997; Shimizu, Tsunogai, & Arai, 1997; Artursson, Palm, & Luthman, 2001).

3.2 ACE inhibitory activity of samples taken from transport assays

Dry-cured ham extracts and peptide solutions taken from the apical and basal compartments at different transport times were analysed for ACE inhibitory activity ~~, directly after sampling and in basal samples also after concentrating four times~~ (Figure 2). In the case of dry-cured ham extracts, s~~S~~amples taken from the apical side showed high ACE inhibition, ~~generally~~ reaching the maximum values at 15 min of cellular transport except for sample S1, which presented slightly higher ACE inhibition before incubation than during the cellular assay. ~~in both types of samples, and with values very close to 100% inhibition for the purified peptides~~. These high inhibition values at 15 min of the transport may be due to a strong action of cell peptidases on these peptides ~~at this time~~, whereby the modification of the peptide structure may affect its biological activity and peptide fragments released can show even a higher degree of ACE inhibitory activity ~~compared to~~in comparison with the precursor peptides (Miguel, Recio, Gómez-Ruiz, Ramos, & López-Fandiño, 2004). In fact, ~~In the case of dry-cured ham samples,~~ samples M1 and S1 showed similar inhibition results even though sample S1 contained only the fractions ~~which~~ showing the highest ACE inhibitory activity. Meanwhile, the activity decreases more significantly after 30 min of transport for samples M2 and M3, which come from dry-cured hams with longer curing process. The

ACE inhibition activity for sample M2 before the transport assay was very low (with a value of 4.5%), which is in agreement with a previous study (Escudero et al., 2014)~~,~~ and ~~which~~ that is probably ~~due~~ owing to this sample has not been deproteinised ~~compared to~~in comparison with sample M3. Additionally, sample M2 did not show any ACE inhibitory activity in the apical side after 60 min of cellular transport, indicating that inhibitory peptides were either completely hydrolysed or transported to the basal side. On the other hand, little to no ACE inhibition was detected in the basal samples, so ~~For this reason, all basal samples~~they were concentrated four times to check if samples may show ACE inhibitory activity at higher concentration. Under these conditions, dry-cured ham extract samples showed activity although to a varying degree, obtaining values around 30-40% at 15 min of transport and then decreasing during time.

With regard to purified peptides, Figure 2 evidences ACE inhibition values very close to 100% in apical samples at 15 min of the transport. ~~On the other hand, little to no ACE inhibition was detected i~~In ~~the~~ basal samples, ~~except~~ significant results were only found for peptides AAPLAP and KPVAAP after 60 min of transport, whereby ACE inhibitory activity reached values of 42% and 30%, respectively. However, ~~for purified peptides~~in the case of concentrated basal samples, the ACE inhibitory activity of peptides AAATP and AAPLAP increased during incubation, reaching values up to 30% for AAATP and 70% for AAPLAP at 60 min of the assay. H~~Also h~~igh percentages of inhibition (around 65%) were also observed for KPVAAP after 15 and 60 min of cellular transport.

These results indicate that some peptides ~~as well as~~along with small derived fragments could be absorbed, maintaining their ACE inhibitory activity after cellular transport although at low concentration in non-concentrated basal samples (Figure 2). Moreover, synergic effects between antihypertensive peptides could explain higher values of ACE

inhibition for peptide mixtures in comparison to the individual activity of ~~individual~~ peptides. In addition, it should be ~~stressed~~ highlighted that the concentration of peptides in the basal and concentrated basal samples does not show a linear but exponential response when ACE activity is plotted against the concentration of ~~an~~ ACE inhibitory peptide~~s,~~ as previously described by Sentandreu and Toldrá~~,~~ (2006).

Because angiotensin I converting enzyme is present in the brush border contributing to hydrolysis of peptides (Yoshioka, Erickson, Woodley, Gulli, Guan, & Kim, 1987), control samples containing only the Caco-2 cells and PBS were also analysed for ACE inhibitory activity, showing non-significant effects (data not shown). Nevertheless, these values were taken into account for determining the percentage of ACE inhibition of the samples.

3.3 Peptide degradation by epithelial peptidases

As dry-cured ham samples contain a very complex mixture of peptides, only the degradation of the purified peptides on the brush-border side was evaluated by MS-based techniques in order to detect those peptides that were transcellularly transported. Table 1 shows the peptide sequences detected by MALDI-ToF/ToF MS in the apical and basal compartments at the different assayed times.

The analysis revealed the degradation of the intact peptide AAATP after 15 min of transport ~~with~~ and the generation of smaller sized peptides, ~~some of~~ which ~~can~~ could cross the intestinal barrier up to the basal side. On the other hand, AAPLAP was detected in the apical chamber throughout the experiment, but analysis of the basal solutions showed that it was not transported intact. In fact, several shorter fragments were also detected in the apical and basal solutions, suggesting its partial degradation by brush border peptidases. Furthermore, peptide KPVAAP was detected up to 60 min of transport in both apical and basal samples, indicating that its chemical and structural

properties allow it to be absorbed intact through the Caco-2 monolayer. However, it was also susceptible to peptidases and different derived fragments were also detected in apical and basal solutions. In this ~~sense~~regard, among all the fragments detected in either apical or basal samples, several of them were previously reported as ACE inhibitory peptides (Table 1 of Supplementary material~~2~~)~~, being LAP and VAA those with the lowest IC_{50} values~~. The remaining peptides detected at different times of incubation as shown in Table 1 (AATP, ATP, AAAT, APLA, PLAP, AAPL, VAAP, KPV, AAA, and VA) were synthetised and assayed for the *in vitro* ACE inhibitory activity in order to calculate their IC_{50} values (Table ~~3~~2). These results highlight the potent inhibition of fragment VAAP resulting from KPVAAP degradation, and PLAP from AAPLAP, which showed IC_{50} values of 16.75 and 76.5 µM, respectively. This fact could be explained by the presence of hydrophobic amino acids such as proline and alanine close to the C-terminal position of the peptides that positively influence the binding to ACE (Rohrbach, Williams, & Rolstad, 1981; Majumder, & Wu, 2009). Hence, the ACE inhibitory activity shown by the peptide fragments could also explain the inhibitory activity found previously in apical and basal samples (see Figure 2-b), suggesting that those fragments transported across the monolayer could also reach the blood stream to exert an antihypertensive activity. As such, brush border peptidases play a key role in the formation and degradation of bioactive peptides and therefore, in their bioavailability and physiological effect (Pihlanto-Leppälä, 2000; Vermeirssen, et al., 2005; Miguel & Aleixandre, 2006).

The study done by Escudero et al. (2013) showed *in vivo* ACE inhibitory activity for the peptide AAATP, although according to the present study it is not absorbed intact through the intestinal barrier (Table 1). These results suggest that either the fragments derived from its degradation are responsible for the decrease in blood pressure in the

SHR model, or AAATP may exert antihypertensive activity through the interaction with receptors expressed in the gastrointestinal epithelia (Yamada, Matoba, Usiu, Onishi, & Yoshikawa, 2002), thereby inducing other mechanisms of blood pressure regulation such as ~~through~~ nitric oxide or endothelin production (Lifton, Gharavi, & Geller, ~~et al.,~~ 2001).

The stability of peptides to enzymatic hydrolysis and absorption processes determine their bioavailability and bioactivity, being the main cause of differences found between *in vitro* and *in vivo* assays (Pihlanto-Leppälä, 2000; Vermeirssen, Van Camp, & Verstraete , 2004; Bejjani, & Wu, 2013). In fact, the amount of peptides absorbed *in vivo* could be higher than *in vitro* assays when Caco-2 cells are used as models~~, due~~ owing to the lower expression of some intestinal transporters and tighter junctions in Caco-2 cell monolayers ~~compared to~~in comparison with *in vivo* intestinal tissues (Lennernäs, Palm, Fagerholm, & Artursson, 1996; Boisset, Botham, Haegele, Lenfant, & Pachot, 2000; Vermeirssen et al., 2005). Nevertheless, this study suggests a beneficial impact of dry-cured ham peptides towards blood pressure due to their potential to exert an *in vivo* antihypertensive action.

4. Conclusions

Previous studies reported the ACE inhibitory activity of some dry-cured ham peptides and their stability to *in vitro* digestion. However, antihypertensive peptides need to resist the complete hydrolysis by brush-border peptidases and be absorbed actively across the intestinal epithelium to exert their activity. The transepithelial transport through a Caco-2 cell monolayer has been studied in dry-cured ham extracts and purified peptides, evaluating the degradation of peptides by mass spectrometry. Results showed that peptides AAATP, AAPLAP, and KPVAAP are degraded throughout the

transport assay, although KPVAAP can also be absorbed intact through the intestinal barrier. Moreover, this study evidences for the first time the absorption and generation of ACE inhibitory peptide fragments originating from dry-cured ham by the Caco-2 cell line. The antihypertensive action of the peptides or small fragments derived as well as the synergic effect between them could explain the ACE inhibitory results obtained in apical and basal samples, suggesting that dry-cured ham peptides could reach the circulatory system to exert an antihypertensive action.

Acknowledgements

The research leading to these results received funding from the European Union 7th Framework Programme (FP7/2007-2013) under Grant Agreement 312090 (BACCHUS). This publication reflects only the author views and the Community is not liable for any use made of the information contained therein. Grant AGL2013-47169-R from MINECO and FEDER funds and the FPI Scholarship BES-2011-046096 from MINECO (Spain) to M.G. are fully acknowledged. The JAEDOC-CSIC postdoctoral contract to L.M. co-funded by the European Social Fund, and BOF (Special Research Fund of Ghent University) for their financial support (project 01B04212) are also acknowledged. The proteomic analysis was carried out in the SCSIE_University of Valencia Proteomics Unit, a member of ISCIII ProteoRed Proteomics Platform.

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FIGURE CAPTIONS

Figure 1. MALDI-ToF mass spectra of samples taken from the transepithelial transport (apical and basal sides) through Caco-2 cell monolayer at different times (0, 15, 30, 60 min) after adding the purified peptides a) AAATP, b) AAPLAP, and c) KPVAAP.

Figure 2. ACE inhibitory activity (%) observed in the apical, basal, and four times concentrated basal compartments obtained from the cellular transport after the application of a) dry-cured ham extracts and b) purified peptides. ~~Different~~Bar letters ~~in~~ indicate significant differences at $p < 0.05$ (n=3) between different times of the cellular transport (0, 15, 30, 60 min) in each compartment and sample (a = apical; **a** = basal 4x; *a* = basal).

~~sample and compartment indicate significant differences at $p < 0.05$ (n=3).~~

Table 1. Peptide sequences detected by using MALDI-ToF/ToF MS in the cellular transport assay.

Precursor peptide	Peptide fragments[a]	Monoisotopic mass (Da)[b]	Apical – times (min)[c]				Basal – times (r	
			0	15	30	60	15	30
AAATP*		429.22	x	x				
	AATP	358.19		x	x	x		
	AAAT	332.17						x
	ATP	287.15		x			x	x
	AAA	231.12						x
AAPLAP*		538.31	x	x	x	x		
	PLAP	396.24		x	x	x		
	APLA	370.44					x	x
	AAPL	370.22					x	x
	PL *	228.15					x	x
	LA *	202.13						
KPVAAP*		581.35	x	x	x	x	x	x
	VAAP	356.21			x		x	x
	KPV	342.23			x		x	x
	KP*	243.16		x		x		
	VA	188.12		x	x	x	x	x
	AP*	186.10		x	x	x		

*Sequences previously identified showing ACE inhibitory activity according to BIOPEP database (se

[a] Possible fragments derived from the degradation of the precursor peptide.

[b] Monoisotopic molecular mass in Daltons of the matched peptide.

[c] Peptides detected in the apical compartment at different transport times.

[d] Peptides detected in the basal compartment at different transport times.

Table 2. ACE inhibitory activity (IC_{50}) of synthetic peptides.

Peptides	IC_{50} (μM)
VAAP	16.75
PLAP	76.50
AAA	111.47
AATP	300.74
ATP	406.56
AAAT	513.65
VA	607.96
KPV	> 1000
AAPL	> 1000
APLA	> 1000

Figure 1

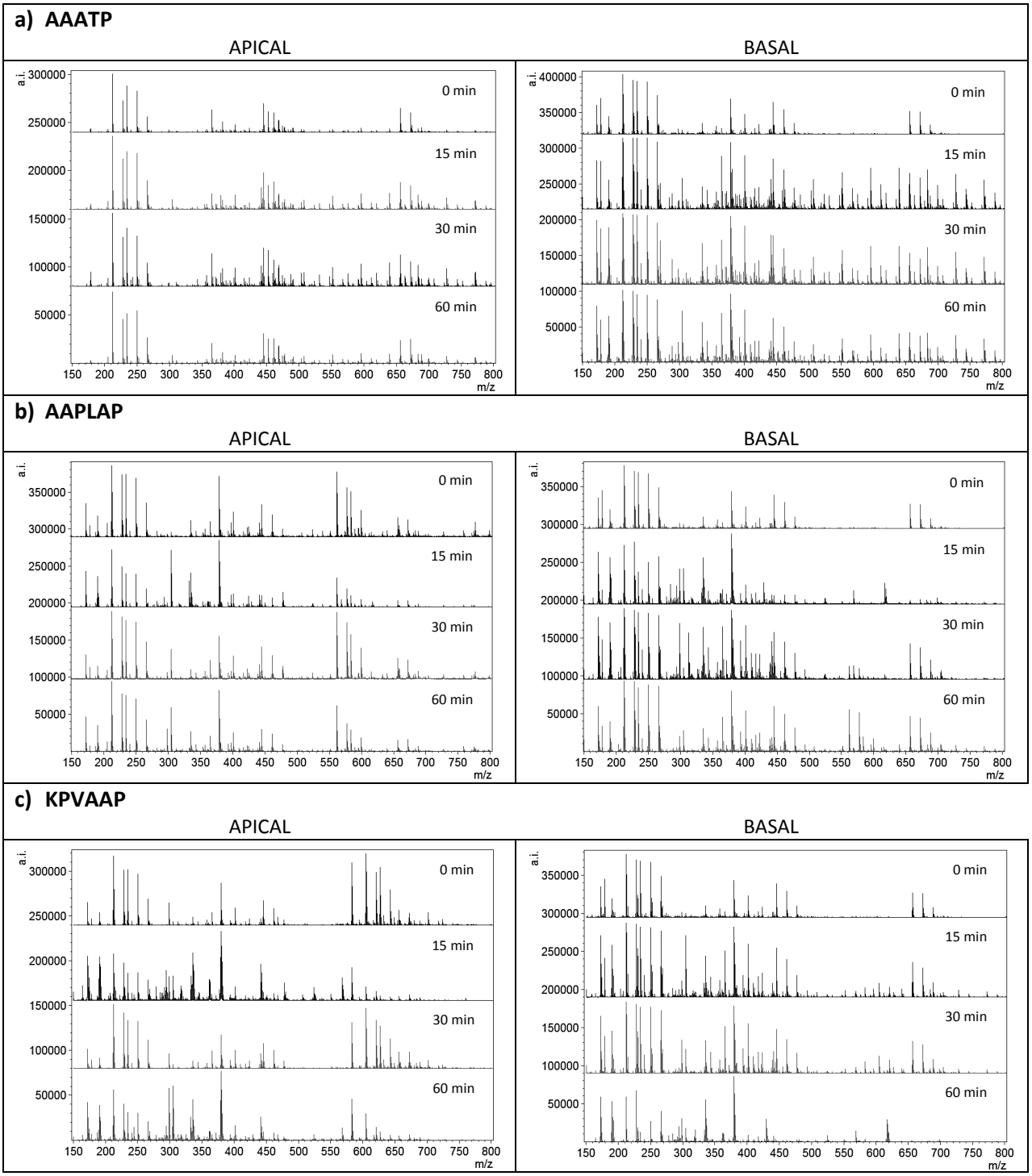
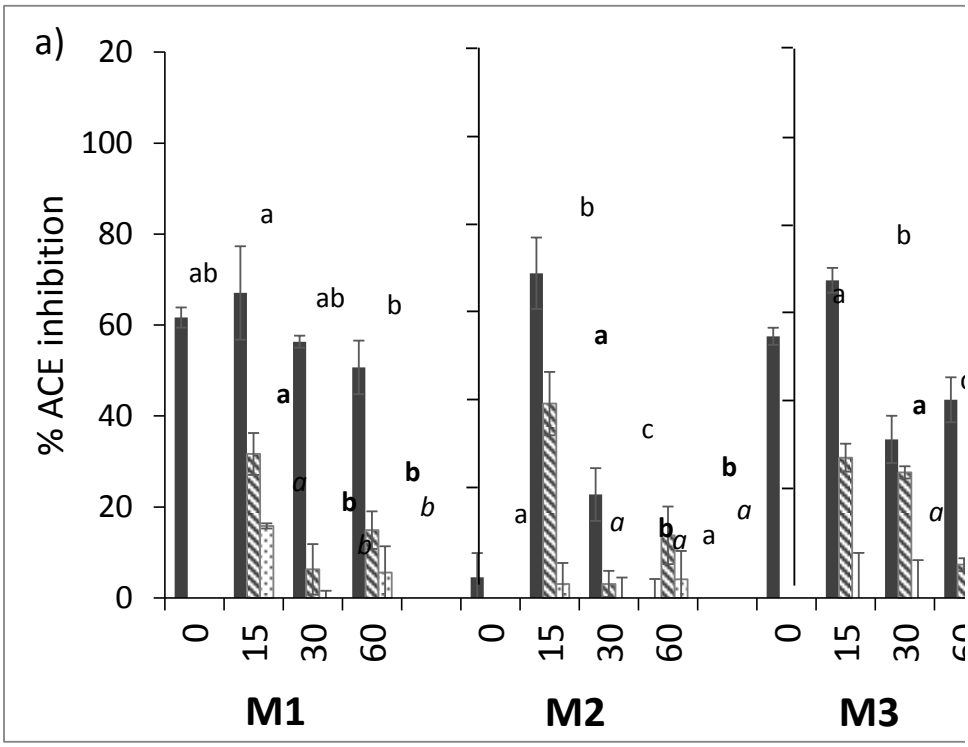


Figure 1.

Figure 2



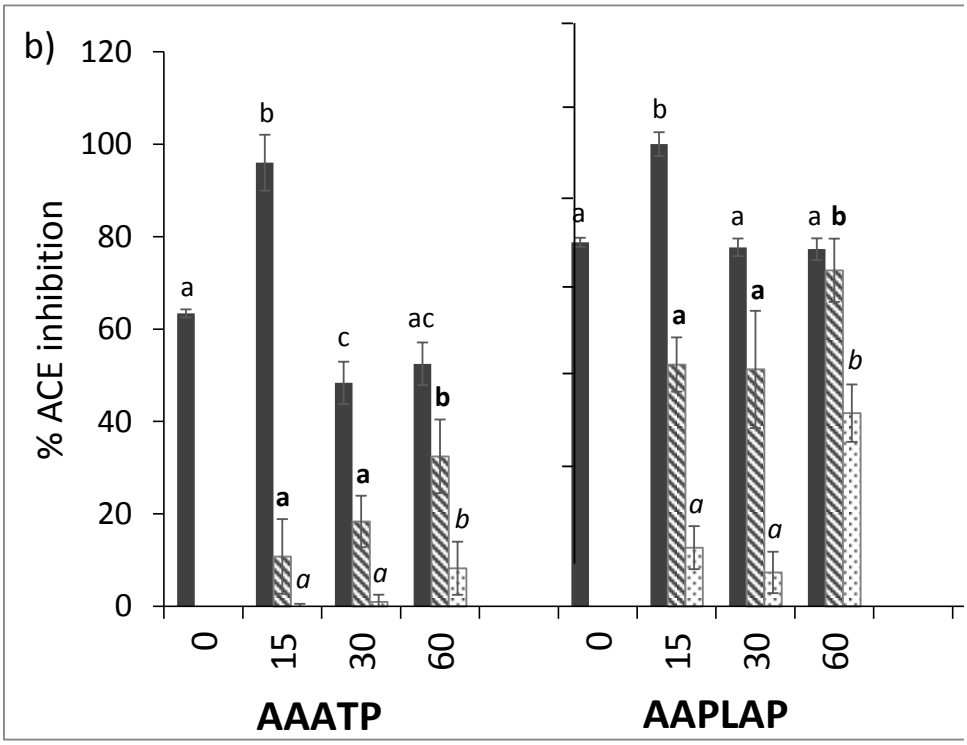


Figure 2.

Supplementary material

Supplementary Table 1. Pep

Peptides	IC_{50} (μM)
KPVAAP	12.37
AAPLAP	14.38
KP	22
AAATP	100
AP	230
LA	310
PL	337.32