Angiotensin I-Converting Enzyme Inhibitory Peptides Generated from in Vitro Gastrointestinal Digestion of Pork Meat

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The main purpose of this work was to study the generation of Angiotensin I-converting enzyme inhibitory (ACEI) peptides after gastrointestinal digestion of pork meat by the action of pepsin and pancreatin at simulated gut conditions. The hydrolysate was further subjected to reverse phase chromatography in order to separate the fractions with ACEI activity. Using MALDI-TOF/TOF mass spectrometry, 12 peptides were identified in these fractions. It is worth highlighting the novel peptides ER, KLP, and RPR with IC_{50} values of 667 μM, 500 μM, and 382 μM, respectively. Results obtained by MALDI-TOF/TOF mass spectrometry were complemented by a second approach consisting of the analysis of the hydrolysate directly by nanoLC-ESI-MS/MS followed by a study of the obtained sequences and comparison with known ACEI peptide sequences. By using these two approaches, a total of 22 peptides were selected for its synthesis and further in vitro assay of ACEI activity. The strongest ACE inhibition was observed for peptide KAPVA (IC_{50} = 46.56 μM) followed by the sequence PTPVP (IC_{50} = 256.41 μM). Sequence similarity searches revealed that these two peptides derive from muscle titin, constituting the first identified ACEI peptides coming from this protein. This is also the first time that ACEI sequences MYPGIA and VIPEL have been reported. Other identified and synthesized sequences showed less ACEI activity. The obtained results evidence the potential of pork meat proteins as a source of antihypertensive peptides after gastrointestinal digestion.

KEYWORDS: Angiotensin I-converting enzyme; pork meat; skeletal muscle; peptides; mass spectrometry; protein digestion; hypertension; proteomics

INTRODUCTION

Among the different types of meat, pork meat is the one with the highest rate of consumption in Spain, being used to produce an extensive variety of processed meat products. Furthermore, pork meat is an important source of high-quality proteins, such as actin, myosin, and collagen, which are the most abundant (*1*).

Many studies have recently demonstrated that pork meat can be used to obtain biologically active peptides that can regulate numerous processes in the organism (*2*). This is important because it gives an added value to pork meat consumption. Peptides have shown to exert antioxidant, antimicrobial, or antihypertensive activities, for example (*3*). Peptides show antihypertensive activity because they are able to inhibit Angiotensin I-converting enzyme (ACE; EC 3.4.15.1). ACE is an enzyme of the rennin−Angiotensin system, a major regulator of cardiovascular and renal function (*4*). It catalyzes the conversion of inactive decapeptide Angiotensin I into a potent vasoconstrictor, the octapeptide Angiotensin II. It also inactivates bradykinin, which reduces blood pressure (*5*). The greater the ACE activity, the more Angiotensin I is converted into Angiotensin II, giving rise to an increase in blood pressure.

Previous studies (*6*) found that enzymatic hydrolysates of porcine skeletal muscle proteins exhibit potent ACEI activity, identifying some of the peptides present in these hydrolysates. These peptides also showed antihypertensive activities in vivo (*7*).

Protein digestion generates many peptides in the gut lumen, and some of these peptides have been shown to possess biological effects (*8*). These bioactive peptide sequences remain inactive within the original protein, but once released, they can function as regulatory compounds. Their activity is directly related to their amino acid sequence. To exert a potential antihypertensive effect after oral administration, ACEI peptides have to reach the cardiovascular system in an active form (*9*). Therefore, they need to remain active during digestion by human peptidases and be transported through the intestinal wall into the bloodstream. It is known that peptides of different length can be absorbed intact from the gastrointestinal tract (*8*).

Efforts made in the study of antihypertensive peptides derived from pork meat hydrolysates are important because they could help to change the negative image that pork meat consumption is bad for our health because of the excess animal fat intake and the effect on our cardiovascular system. However, bioactive peptides

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derived from pork meat hydrolysates could be utilized as functional ingredients to develop new healthy meat products or to produce nutraceuticals in supplemented form.

In the present study, we investigated the ACEI activity derived from peptides released by enzymatic hydrolysis after simulating human digestion of porcine skeletal muscle proteins. The peptides generated from pork meat during in vitro digestion were sequenced by using both MALDI-TOF/TOF and by nanoLC-ESI-MS/MS mass spectrometry.

MATERIALS AND METHODS

Materials. Fresh pork skeletal muscle (*Longissimus dorsi*) was obtained from Industrias Cárnicas Vaquero S.A (Madrid, Spain). Pepsin (from hog stomach) was purchased from Fluka Chemie Gmbh (Buchs, Switzerland), and pancreatin (from porcine pancreas) and Angiotensin-converting enzyme (from rabbit lung) were 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). Other chemicals were from Sharlau Chemie S.A (Sentmenat, Spain).

In Vitro Digestion of Porcine Muscle Proteins. Human digestion of muscle proteins was simulated in vitro using pepsin and pancreatin according to the method of Laparra et al. (*10*), with some modifications. For this purpose, 4 g of raw pork meat (*Longissimus dorsi*) was suspended in 40 mL of bidistilled water. After simulating human chewing by using a Stomacher for 1 min, the pH was adjusted to 2 with 6 M HCl. After that, pepsin was added at a 1:100 enzyme to substrate ratio.

After 2 h of digestion at 37 °C 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 °C, enzyme activity was terminated by heating for 10 min at 95 °C. The reaction mixture was centrifuged at 10.000 rpm for 20 min, and the resulting supernatant was used for the separation and identification of peptides.

Porcine muscle protein hydrolysate was deproteinized by adding 3 vol of methanol while maintaining the sample for 20 min at 4 °C. After that, the sample was centrifuged (24500g for 20 min at 4 °C), and the supernatant was dried using a rotatory evaporator. The dried deproteinized hydrolysate was redissolved in 1 mL of 0.1% trifluoroacetic acid (TFA) in bidistilled water.

Purification of ACE Inhibitory Peptides. This extract was fractionated by reverse phase chromatography using an Agilent 1100 HPLC system (Agilent Tech., California, USA) equipped with a quaternary gradient pump, an in-line degasser, a diode array absorbance detector, and an autosampler (all 1100 series, Agilent Tech., Waldbronn, Germany). The column used in this experiment was a Symmetry C18 (4.6 × 250 mm, 5 μm) from Waters Co. (Milford, MA). The injection volume was 100 μL. Solvent A was 0.1% TFA, and solvent B contained 0.085% TFA in 60% acetonitrile (ACN)/40% bidistilled water. Peptides were first eluted with an isocratic gradient of 99% solvent A for 5 min, followed by a linear gradient from 1% to 100% of solvent B in 80 min at a flow rate of 0.8 mL/min. Fractions (0.8 mL) were collected and assayed for ACE inhibitory activity. Those fractions showing remarkable ACE inhibition were further analyzed by MALDI-TOF/TOF mass spectrometry in order to identify the peptides contained in them.

Assay of ACE Inhibitory Activity. The ACE inhibitory activity of the different fractions and identified peptides was measured according to the method developed by Sentandreu and Toldrá (*11*). 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) was mixed with 50 μL of 150 mM Tris-base buffer (pH 8,3) containing 3 mU/mL of ACE, then preincubating the mixture 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 10 min at 37 °C. 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. The concentration of each peptide required to inhibit 50% of ACE activity was defined as the IC_{50} value.

Molecular Mass Determination and Peptide Sequencing by MALDI-TOF/TOF Mass Spectrometry. Identification of the sequence of peptides present in fractions exhibiting remarkable ACEI activity was carried out by matrix assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF MS). Each fraction was dried in a vacuum centrifuge and resuspended in 7 μL of 0.1% TFA, and 1 μL was spotted onto a MALDI plate. After the droplets were air-dried at room temperature, 1 μL of matrix (5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) in ACN/H₂O (1:1, v/v) containing 0.1% TFA) was added and allowed to air-dry at room temperature. Both MS and MS/MS data were acquired with a 4700 Applied Biosystems MALDI-TOF/TOF mass spectrometer (Foster City, CA). MALDI-TOF analysis was done covering a mass range of 300−1500 Da. Automatically, main ions were selected for the subsequent TOF/TOF analysis. External calibration of the instrument was performed using 4700 Cal Mix (Applied Biosystems) according to manufacturer's indications. For MS/MS calibration, the fragmentation of Angiotensin I included in the 4700 Cal Mix was used.

Separation and Peptide Identification by nano LC-ESI-MS/MS. Results obtained by MALDI-TOF/TOF MS were complemented by analysis of the deproteinised protein hydrolysate. A LC-ESI-MS/MS analysis were performed using an Ultimate nanoLC system (LC Packings, Amsterdam, The Netherlands) coupled to a nanoelectrospray ion source (Protana, Odense, Denmark) and a QSTAR XL Q-TOF hybrid mass spectrometer (MDS Sciex, Applied Biosystems, Concord, Canada). Sample (5 μL of the initial protein hydrolysate is diluted to 60 μL with 0.1% TFA) was delivered to the system using a FAMOS autosampler (LC Packings) at 40 μL/min, and the peptides were trapped onto a PepMap C18 precolumn (5 mm × 300 μm i.d.; LC Packings). Peptides were then eluted onto a PepMap C18 analytical column (15 cm × 75 μm i.d.; LC Packings) at 300 nL/min and separated using a linear gradient of 5−50% solvent B (95% ACN in 0.1% TFA, v/v) in 120 min. The QSTAR XL was operated using an information-dependent acquisition mode, in which a 1-s TOF MS scan from 100 to 1000 *m/z*, was performed, followed by 3-s product ion scans from 100 to 1000 *m/z* on the most intense ions. The QSTAR XL Q-TOF was calibrated with a mixture of CsI and cPDI inhibitor.

Automated spectral processing, peak list generation, and database search were performed using Mascot search v1.6b21 script for Analyst QS 1.1 (Applied Biosystems, Foster City, CA) in combination with the Mascot search engine (Matrix Science, Inc., London, U.K.) and ProteinPilot 2.0.1 software (Applied Biosystems, Foster City, CA) with Paragon algorithm. Identification of the protein origin of peptides was performed using the National Center for Biotechnology Information (NCBI) nonredundant protein database. Automated matches of MS/MS spectra against peptide sequences contained in the database were subsequently verified manually. NanoLC-ESI-MS/MS analysis of the deproteinized protein hydrolysate was done in triplicate in order to validate results and confirm the peptide sequences.

Peptide Synthesis. ACE inhibitory peptides identified from pork meat hydrolysate were synthesized by Gen Script Corporation (Piscataway, NJ, USA) in order to perform in vitro inhibition of ACE activity. The purity of the synthesized peptides was certified by analytical LC-MS.

RESULTS AND DISCUSSION

Isolation and ACE Inhibitory Activity of Fractions Obtained from Pork Muscle Extract. The final hydrolysate obtained by sequential incubation with pepsin and pancreatin was fractionated by reversed-phase chromatography. During the chromatographic separation (**Figure 1**), 0.8 mL fractions were collected and analyzed separately for ACE inhibitory activity. After separation, fractions were dried and redissolved in 70 μL of 150 mM Tris-base buffer (pH 8.3), assaying ACEI activity as previously described. Fractions eluting between 4 and 34 min had a relatively high peptide concentration and showed ACEI values ranging from 22% to 77%. We did not discard small peptides eluted in the first part of the chromatogram because they could also be potentially active. Fraction 6 yielded the highest ACE inhibition (77%) possibly due to the presence of short peptides. Fractions 11, 14,
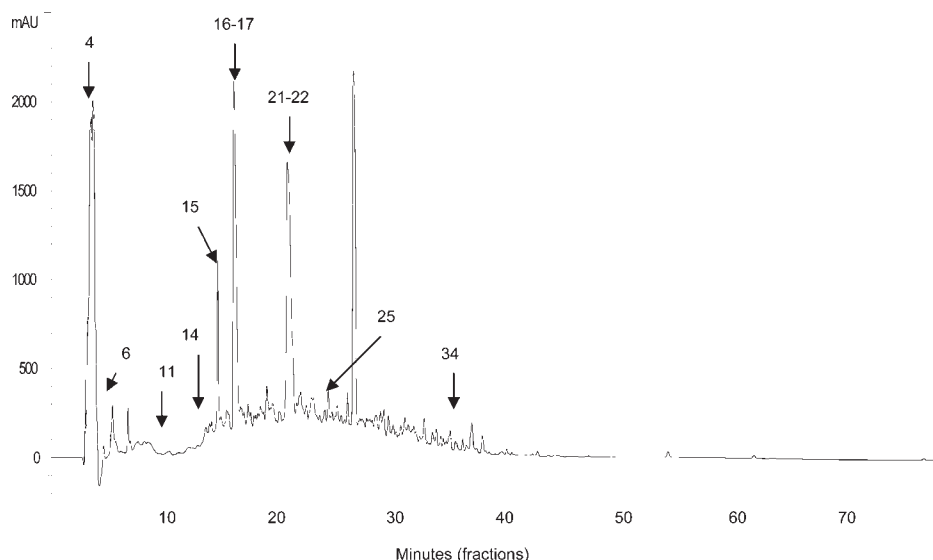
Figure 1. Reversed-phase chromatographic separation of the pork meat hydrolysate. Peak numbers indicate the collected fractions showing ACEI activity.

Table 1. Peptides Separated from the Pork Muscle Hydrolysate by Reversed-Phase Chromatrography (**Figure** 1) and Further Identified by MALDI-TOF/TOF[a]

fraction	calc mass	obs mass	sequence	parent protein	NCBI accesion no.	position	IC$_{50}$ (μM)
6	303.1	304.1	ER	protein-lysine 6-oxidase	P45845	23−24	667
	400.2	401.2	EPR	RNA binding protein	XP 001924460	15−17	>1000
	400.2	401.2	PER	skeletal muscle gated chloride channel	AAC48666	655−657	>1000
11	356.2	357.2	KLP	DNA polymerase α-catalytic subunit[b]	XP 242396	817−819	500
	356.2	357.2	AGLP	collagen type IV alpha-6	XP001925433	365−368	>1000
	328.1	328.1	GPR	opimelanocortin N-term G1	0811187A	41−43	>1000
	387.2	387.2	NVR	mono (ADP-ribosyl) transferase[c]	CAC 69966	61−63	>1000
	328.1	328.1	PGR	chain A collagen type I[d]	1y0F	76−78	>1000
14	427.2	428.2	VGPR	collagen type VII alpha-1	XP001924460	1622−1625	n.a[e]
	427.2	428.2	RPR	nebulin	XP001927009	1263−1265	382
17	496.2	497.2	PAGPR	collagen type VII alpha-1	XP001924460	2765−2769	n.a
	496.2	497.2	PAGPVG	collagen type V alpha-1	NP001014971	562−567	n.a

[a] In vitro ACEI activity of synthetic peptides is expressed as IC$_{50}$ (concentration of peptide needed to inhibit 50% of the original ACE activity). All of the sequences except those indicated with superscripts were found in proteins of the porcine species. [b] Parent protein from *Rattus norvegicus*. [c] Parent protein from *Lemur catta*. [d] Parent protein from *Rattus norvegicus*. [e] No ACEI activity was found.
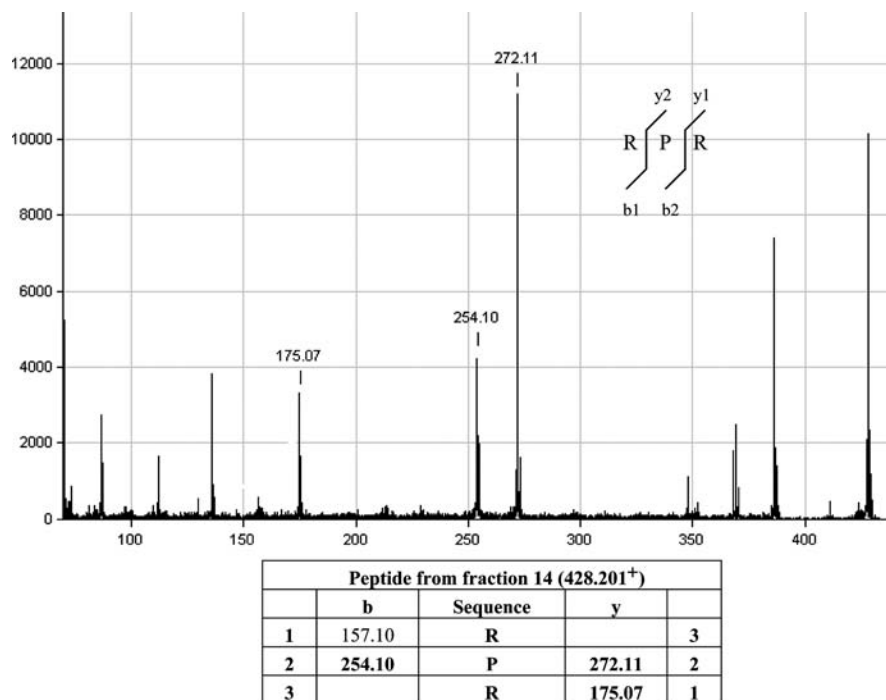
and 16−17 showed percentages of ACE inhibition of 57.03%, 22.11%, and 32.27%, respectively. The other collected fractions indicated in **Figure 1** did not exert a remarkable inhibition. It has been suggested that ACE inhibitory peptides are rich in hydrophobic amino acids, which results in a medium to high retention on C18 reverse phase columns (*12*).

Fractions showing ACE inhibition were further analyzed separately by mass spectrometry with the aim to characterize their peptide content.

Peptide Sequencing Using Mass Spectrometry. In a first approach for the identification of ACE inhibitory peptides, the most potent ACEI fractions obtained after reversed-phase chromatography were subjected to MALDI-TOF/TOF mass spectrometry in order to determine both molecular masses and sequence of the peptides contained in them. A total of 33 peptide sequences were identified, but only peptides with the size and structural requirements for ACE inhibition were synthesized and their IC$_{50}$ calculated (see **Table 1**). All spectra were interpreted both manually and by using the online form of the Mascot search engine. BLAST sequence similarity searches revealed 100% homology with some of the identified sequences with pork proteins, but some other peptides showed homology with proteins from other

species (**Table 1**). This can be explained by the fact that all pork proteins have not been completely identified and introduced in protein databases, but it is believed that these peptides may have also been originated from pork. **Figure 2** shows the MS/MS spectrum corresponding to peptide RPR as an example of the quality of the obtained spectra.

It is known that some of the most potent ACE inhibitory peptides are small peptides (*13*). In this respect, we have identified three novel peptides with relatively high inhibitory activity. The dipeptide ER yielded an IC$_{50}$ value of 667 μM, whereas tripeptides KLP and RPR showed IC$_{50}$ values of 500 μM and 382 μM, respectively. ACE binds preferably to peptides with hydrophobic (aromatic or branched-chain aliphatic) amino acid residues at each of the three positions closest to the C-terminus. KLP ends with proline, one of the most favorable C-terminal amino acids for binding to the ACE active site (*14*). It has been proposed that the positive charge on the ε-amino group of the C-terminal arginine or lysine side chain also contributes to inhibitory ACE activity (*5*). The peptides ER and RPR both end with arginine, which could explain their inhibitory activities. The exceptionally strong ACE inhibitory activity found in some fractions of the pork

Peptide from fraction 14 (428.201[+])				
	b	**Sequence**	**y**	
1	**157.10**	R		3
2	**254.10**	P	**272.11**	2
3		R	**175.07**	1

Figure 2. MS/MS spectrum of ion 428.20[1+] present in fraction 14 of the reverse-phase chromatograph. Peptide sequence matching the product ion spectrum (RPR) is shown in capital letters. The spectrum is presented with its corresponding table showing b and y ions matched by MASCOT in bold letters.
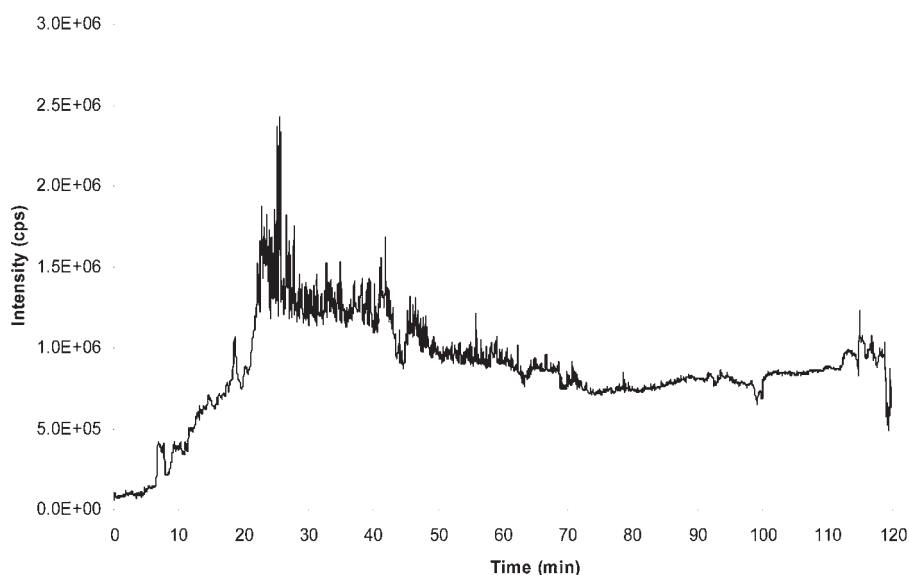
Figure 3. Chromatogram corresponding to the separation of the pork meat hydrolysate by nanoLC-ESI-MS/MS using a QSTAR XL Q-TOF hybrid mass spectrometer.

meat hydrolysate could indicate a possibly synergistic effect among various peptides, as has been already reported (*15*).

The activities of these novel peptides were lower than that of some of the previously identified peptides from different protein sources (*16−18*), but they were comparable to those previously identified from pork meat hydrolysates (*6, 19*). These previously reported peptides have not been identified in our digests, which may be due to differences in sample preparation or in the different technique used for the identification of the peptide sequences. To our knowledge, the bioactive sequences reported in this work have not been described before.

As a complement to results obtained by MALDI-TOF/TOF mass spectrometry and with the aim to cover more information about AC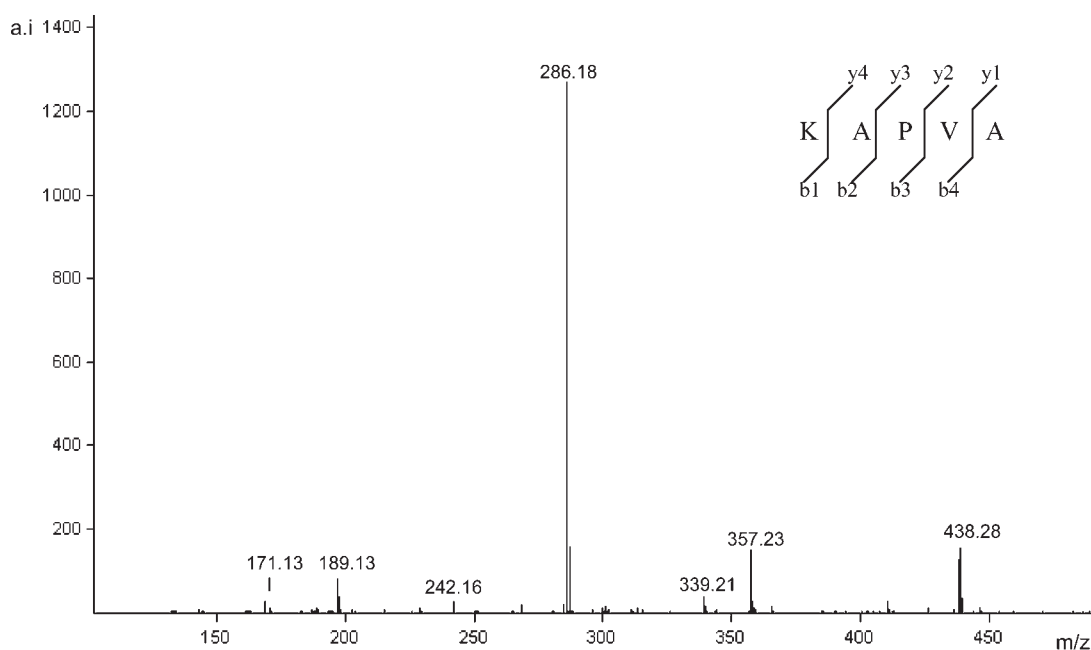E inhibitory peptides coming from pork meat, the protein hydrolysate was further analyzed by liquid chromatography coupled to a quadrupole time-of-flight mass spectrometer equipped with a nanoelectrospray ionization source (nanoLC-ESI-QTOF MS). The peptide separation profile obtained by this way is shown in **Figure 3**. Using this approach, a large number of peptides were identified, including some of the sequences identified by MALDI-TOF/TOF. After an exhaustive analysis of all of the information provided by this second analysis, only those peptides with the size and structural requirements adequate for ACE inhibition were synthesized and their IC$_{50}$ calculated (see **Table 2**). All MS/MS spectra were interpreted both manually and by using the online form of the MASCOT search engine and the Paragon algorithm. As an example, **Figure 4** shows the MS/MS spectrum corresponding

Table 2. Identification of Peptides Present in the Porcine Protein Hydrolysate by LC-ESI-MS/MS[a]

retention time	calc mass	obs mass	sequence	porcine parent protein	NCBI accesion no.	position	IC_{50} (μM)
25.2	589.29	589.96	MMVPI	cytochrome P450 3A46	NP001128296	394–398	>1000
28.54	445.26	446.26	IGGSI	beta-actin	ABI29185	343–347	na[b]
31.6	526.31	527.32	KAPVA	titin	XP001925902	4784–4788	46.56
35.43	509.28	511.33	PTPVP	titin	XP001925902	4216–4220	256.41
36.01	519.27	520.28	YPGIA	beta-actin	ABI29185	308–312	na
36.89	526.32	527.32	NIIPA	GAPDH[c]	ABI29187	203–207	>1000
47.18	650.32	651.33	MYPGIA	beta-actin	ABI29185	307–312	641.02
59.06	569.34	570.35	VIPEL	GAPDH	ABI29187	218–222	799.24
60.35	603.35	604.36	INDPF	GAPDH	ABI29187	31–35	na
64.87	569.34	570.36	VLPEI	titin	XP001925902	4316–4320	>1000

[a] The sequences were further synthesised, and in vitro ACEI activity was determined and expressed as IC_{50} (concentration of peptide needed to inhibit 50% of the original ACE activity). All analysis were done in triplicate. [b] ACEI activity was not found. [c] Gliceraldehyde 3-phosphate dehydrogenase.

Peptide eluted at min 31.6 (527.32[1+])

	b	Sequence	y	
1	**171.13**	**K**		5
2	**242.16**	**A**	**357.23**	4
3	**339.21**	**P**	**286.18**	3
4	**438.28**	**V**	**189.13**	2
5		**A**	90.05	1

Figure 4. MS/MS spectrum of ion 527.32[1+] obtained from the analysis of the pork meat hydrolysate by nanoLC-ESI-MS/MS. Peptide sequence matching the product ion spectrum (KAPVA) is shown in capital letters. The spectrum is presented with its corresponding table showing b and y ions matched by MASCOT in bold letters.

to the peptide KAPVA. This peptide had a retention time of 31.6 min on the nanoLC separation (**Figure 3**).

From this second approach, we synthesized 9 pentapeptides and 1 hexapeptide (**Table 2**). The sequence KAPVA yielded the strongest ACE inhibition, with an IC_{50} value of 46.56 μM. KAPVA has as the C-terminal alanine, which is supposed to play an important role in binding to ACE (20). The presence of proline in C-antepenultimate position also seems to promote the binding to the enzyme (21). The protein origin of KAPVA was titin, also known as connectin, the third most abundant protein of vertebrate

striated muscle after myosin and actin. The titin molecule is formed by a single polypeptide chain with a molecular weight for up to ~4 MDa, constituting the largest protein found in nature so far. The peptide PTPVP also exerted a notable ACE inhibition with an IC_{50} value of 256.41 μM. This peptide has a proline residue in both ultimate and antepenultimate position, titin also being the origin of this peptide. From these results, it can be concluded that titin could be a good source of antihypertensive peptides, KAPVA and PTPVP being the first ACEI peptides identified from this giant protein. The peptide sequences MYPGIA

Table 3. Sequences Identified in the Porcine Protein Hydrolysate by LC-ESI-MS/MS That Share Some Sequence Homology with Previously Described Antihypertensive Peptide Sequences[a]

sequences identified in this work	previously reported sequences	IC_{50} (μM) of the previously reported sequences	ref
RVAPEEHPT	LR**VAPEEHPT**L	n.i[b]	25
VAPEEHPT	LR**VAPEEHPT**L	n.i	25
LFDKPVSPL	**LFDKPVSPL**	n.i	25
FDKPVSPL	**FDKPVSPL**	n.i	25
ITTNPY	**ITTNP**	549	6
MLGQTPT	RM**LGQTPT**K	34	30
DQVFP**MNPPK**	**MNPPK**	945.5	6

[a] Residues in bold letters indicate sequence homology with the peptides found in this study. [b] Not indicated.

and VIPEL showed moderate ACE inhibition (IC_{50} values 641.02 μM and 799.24 μM, respectively) possibly because they contained hydrophobic aminoacids in the C-terminal position such as alanine and leucine, respectively (22) .

It is reported that small peptides are more easily absorbed in the intestinal tract than larger peptides (23) and that peptides containing proline are generally resistant to enzymatic digestion (24). In this respect, we have used two digestive steps (pepsine and the pool of peptidases knows as pancreatin) that have led to obtaining very short peptides which have proline in their sequences as a final product. According to these statements, peptides identified in this work fit well with these characteristics, which would indicate the resistance of these peptides against further digestion and the possibility to be absorbed as such into epithelial cells, thus having the potential to exert an in vivo ACEI activity. Consequently, although in vitro ACEI activities obtained in this study are somewhat moderate compared with those of other ACEI peptides reported in the literature, it is expected that tripeptides and pentapeptides showing ACE inhibition and containing proline residues in their sequences would display remarkable in vivo ACEI activity (7). Since our study has to be considered as a preliminary study, further research is needed to determine potential in vivo antihypertensive activities in spontaneously hypertensive rats.

In addition to the peptides of **Tables 1** and **2**, some larger peptides have been identified after the analysis of the muscle protein hydrolysate by LC-ESI-MS/MS, but they were not further synthesized for determining IC_{50}. Interestingly, these peptides share partial or total homology with peptides that have been previously described in the literature as ACE inhibitors (**Table 3**). Two of these peptides, with the sequences LFDKPVSPL and FDKPVSPL, had been previously found in a study concerning peptides generated during in vivo digestion of proteins from beef and trout by gastric or intestinal enzymes in pigs (25). These previously reported sequences came from muscle creatine kinase, an enzyme involved in the energy metabolism of tissues. It is predictable that these peptides exert ACEI activity due to their bioactive sequences VSP and LF (26, 27). Although the ACEI activity of di- or tripeptides may not always be strictly extrapolated to longer peptides (28), the structural similarity of the C-terminal region may allow a similar activity to be anticipated (29). The sequences RVAPEEHPT and VAPEEHPT share partial homology with the previously reported peptide LRVAPEEHPTL, also released during the digestion of beef and trout proteins (25). These peptides came from actin skeletal muscle, a protein involved in many important cellular processes including muscle contraction, cell motility, cell division, and cytokinesis. These peptides share the bioactive sequence VAP (26); therefore, in the same way they may exert some ACE inhibitory activity. The sequences ITTNPY and DQVFPMNPPK also share partial homology with peptides ITTNP and MNPPK, respectively,

which were previously identified in thermolysin digests of porcine skeletal muscle (6). These peptides came from the porcine myosin heavy chain. Myosin is the major myofibrillar protein in skeletal muscle, making up more than one-third of the total muscle protein content. These two pentapeptides showed IC_{50} values of 549 μM and 945.5 μM, respectively. Moreover, they showed antihypertensive action when administered orally to spontaneously hypertensive rats (7). Finally, the latter peptide MLGQTPT shared partial sequence with the reported peptide RMLGQTPTK. This peptide, which was previously found in a pepsin digest of porcine skeletal muscle troponin, was reported to have an IC_{50} value of 34 μM (30). Troponin comprises three regulatory proteins and is integral to muscle contraction in skeletal and cardiac muscles, but not smooth muscle.

It is worth noting that peptides from **Tables 1**, **2**, and **3** came from the in vitro digestion of major muscle proteins; therefore, we could expect a concentration of the released peptides proportional to the amount of these major proteins after the ingestion of pork meat. From this perspective, we could consider pork meat as a potential source of bioactive peptides.

In conclusion, results reported in this work suggest that physiological digestion of pork meat proteins may promote the generation of bioactive peptides. The developed approach, using two different techniques on mass spectrometry (MALDI-TOF/TOF and LC-ESI-QTOF) allowed us to identify peptides in the digest of pork meat. Very short peptides (2−3 amino acids) were identified using MALDI-TOF/TOF mass spectrometry, whereas peptides from 5 amino acids to quite larger sequences were elucidated by using LC-ESI-QTOF mass spectrometry. To the best of our knowledge, this is the first time that ACE inhibitory peptides have been obtained from the hydrolysis of titin, suggesting that this protein could be a good source of antihypertensive peptides. Other identified and further synthesized peptides also showed remarkable ACEI activity. Some of the identified peptides that were not assayed for ACEI activity showed interesting structural similarities with previously reported peptides showing ACE inhibition. These results would support the idea that pork meat can be a good source of health-promoting constituents that could be potentially used as functional foods or nutraceuticals.

Our study has to be considered a first approach to better understand how proteins from pork meat can be broken down into small fragments and may exert their nutritional function or, in this case, their bioactive function. Nevertheless, further research is needed to determine if these peptides can be effectively transported through the intestinal wall into the bloodstream and exert in vivo antihypertensive activity.

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