JOURNAL OF PROTEOMICS 89 (2013) 183–190

Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jprot

Peptides with angiotensin I converting enzyme (ACE) inhibitory activity generated from porcine skeletal muscle proteins by the action of meat-borne *Lactobacillus*

Patricia Castellano[a,*], *María-Concepción Aristoy*[b], *Miguel Ángel Sentandreu*[b], *Graciela Vignolo*[a], *Fidel Toldrá*[b]

[a]*Centro de Referencia para Lactobacilos (CERELA), Chacabuco 145, 4000 Tucumán, Argentina*
[b]*Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain*

ARTICLE INFO

Article history:
Received 24 April 2013
Accepted 16 June 2013
Available online 25 June 2013

Keywords:
Proteomics
Angiotensin I converting enzyme
Pork proteins
Bioactive peptides
Meat-borne *Lactobacillus*

ABSTRACT

Angiotensin I converting enzyme (ACE) inhibitory activity of peptides derived from the hydrolysis of sarcoplasmic and myofibrillar porcine proteins by the action of *Lactobacillus sakei* CRL1862 and *Lactobacillus curvatus* CRL705 (whole cells + cell free extracts) was investigated at 30 °C for 36 h. The protein hydrolysates were subjected to RP-HPLC in order to fractionate the extracts for further evaluation of ACE inhibitory activity. Bioactive fractions were only found from the hydrolysis of sarcoplasmic proteins by both assayed lactobacilli strains. Identification of peptides contained in the bioactive fractions was carried out by tandem mass spectrometry using a nanoLC-ESI-QTOF instrument and the mascot search engine. From the four most active fractions obtained, a total of eighteen and fifty peptides were characterized from *L. sakei* CRL1862 and *L. curvatus* CRL705 protein hydrolysates, respectively. The sequence FISNHAY was generated by the proteolytic activity of the two lactobacilli species. Sequence similarity analyses between the peptides identified in this study and those previously identified as ACE inhibitory peptides and detailed in the BIOPEP database were outlined. Results suggest that meat-borne *Lactobacillus* were able to generate peptides with ACE inhibitory activity, highlighting their potential to be used in the development of functional fermented products.

Biological significance
The results of this study would enable the obtention of porcine functional foods by applying lactic acid bacteria generating bioactive peptides. ACE inhibitory peptides obtained by the hydrolytic action of *L. curvatus* CRL705 and *L. sakei* CRL1862 on sarcoplasmic proteins were analyzed. Among them, the peptide FISNHAY exhibited the highest activity and its sequence has not yet been reported.

* *Corresponding author*. Tel./fax: +54 381 4311720.
E-mail address: patricia@cerela.org.ar (P. Castellano).

1874-3919
http://dx.doi.org/10.1016/j.jprot.2013.06.023

1. Introduction

Food proteins have long been recognized for their nutritional and functional properties. Nutritional properties are associated with their amino acid content in conjunction with the physiological utilization of specific amino acids upon digestion and absorption [1,2]. On the other hand, the functional properties of proteins are related to their contribution to physiochemical and sensory properties of foods [3]. In recent years, a considerable amount of research has also focused on the release of bioactive peptides which are encrypted within the primary structure of food proteins, in view to investigate such peptides as functional food ingredients aimed at health maintenance.

A variety of biologically active peptides derived from food proteins have been identified [4]. Inhibitors of angiotensin I converting enzyme (ACE) have attracted particular attention for their ability to prevent hypertension. Angiotensin I converting enzyme is a dipeptidyl carboxypeptidase [EC 3.4.15.1] that not only catalyzes conversion of angiotensin I to angiotensin II causing hypertension, but also inactivates bradykinin, a vasodilatory peptide [5]. Consequently, those compounds capable to inactivate the activity of this enzyme would generate the opposite effect, i.e. a decrease of blood pressure. Many ACE inhibitor peptides have been isolated from enzymatically digested food proteins such as casein, whey proteins, plant proteins [6] and muscle sources [7]. On the other hand, proteolytic release of bioactive sequences by lactic acid bacteria (LAB) has been debated recently due to the great advantages of using food grade microorganisms to enrich foods with bioactive substances [8]. As reported in the literature, potent ACE inhibitory peptides were produced during milk fermentation or casein proteolysis by lactic acid bacteria proteases [8–10] however this effect has been poorly documented from meat proteins. Although, a study has been performed on marine proteins, particularly during fermentation of shrimp (*Acetes chinensis*) with *Lactobacillus fermentum* SM605 in which three ACE inhibitory peptides were identified [11].

In this work, *L. curvatus* CRL705 and *L. sakei* CRL1862 isolated from traditional Argentinean sausages [12,13] were used as proteolytic enzyme sources to evaluate the generation of ACE inhibitory peptides from porcine skeletal muscle proteins Muscle foods serve as interesting substrates for investigation into the potential presence of bioactive peptides, specifically ACE inhibitory peptides to design new healthy meat products.

2. Materials and methods

2.1. Bacterial strains and culture conditions

Lactobacillus curvatus CRL705 and *Lactobacillus sakei* CRL1862 strains from CERELA culture collection, previously isolated from traditional sausages [12,13], were used to generate bioactive peptides. The strains were routinely grown in MRS broth (Britania, Argentina) at 30 °C for 18 h and then maintained at −80 °C in 15% (vol/vol) glycerol. Counts were carried out using Plate Count Agar for total mesophiles and MRS agar for LAB at 30 °C during 48 h.

2.2. Preparation of *L. curvatus* CRL705 and *L. sakei* CRL1862 cell suspensions and meat protein extracts

Whole-cell (WC) suspensions (150 ml) from *L. sakei* CRL1862 and *L. curvatus* CRL705 grown to logarithmic phase were collected by centrifugation (10,000 rpm, 20 min, 4 °C), washed twice in 20 mM phosphate buffer (pH 7.0), and resuspended in the same buffer (20% of initial volume). The cell free extracts (CFE) were obtained by the procedure described by Sanz et al. [14]. Meat protein extracts were obtained from porcine *longissimus dorsi* sarcoplasmic and myofibrillar proteins according to Fadda et al. [15]. The protein contents of sarcoplasmic and myofibrillar extracts determined by the method of Bradford [16] using bovine serum albumin as standard were 2.50 and 0.60 mg/ml, respectively. For both extracts, sterility was confirmed by determining the absence of bacterial growth on Plate Count Agar (Merck, Argentina) incubated overnight at 30 °C.

2.3. Protein hydrolysis and peptide analysis

For each meat protein extract (sarcoplasmic or myofibrillar) a combination (1:1) of CFE + WC suspension of *L. curvatus* CRL705 or *L. sakei* CRL1862, added separately, was used as proteolytic enzyme source. The reaction mixture consisted of 3 ml of each suspension (WC and CFE) aseptically added to 30 ml of protein extract. The mixtures were incubated at 30 °C in a shaken water bath and sampled at time 0 h and after 96 h for further analyses. In each case, control samples without the addition of any bacterial enzymes were assayed simultaneously. The extracts with different treatments were centrifuged for 20 min at 14,000 rpm, and the supernatants were collected for the experiments.

The analysis of generated peptides was performed by RP-HPLC as follows: 4 ml of each sarcoplasmic or myofibrillar extract was deproteinized with 10 ml of methanol, centrifuged (10,000 rpm, 10 min, 4 °C), supernatants concentrated by evaporation to dryness and resuspended in deionized water. Peptide extracts were analyzed at day 0 and after 96 h of incubation, using a 1050 high-performance liquid chromatograph (Agilent, Palo Alto, CA, USA) equipped with a photodiode array detector and manual injector. A 4.6 × 250 mm Symmetry C18 column (Waters, Milford, MA, USA) was used. The mobile phase consisted of solvent A (0.1% trifluoroacetic acid in water) and solvent B (acetonitrile–water–trifluoroacetic acid [60:40:0.085, vol/vol/vol]). The elution was performed as follows: an isocratic phase in 1% solvent B for 5 min, followed by a linear gradient from 1 to 100% solvent B for 55 min, at a flow rate of 1.0 ml/min at 40 °C. Peptides were detected at 214 nm.

2.4. Assay for ACE inhibitory activity

The fractions were collected, dried and re-dissolved in 70 μl of 150 mM Tris-base buffer (pH 8.3). The ACE inhibitory activity of each fraction was measured according to procedures described previously [17]. 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-(NO_2)-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 the mixture was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of 200 μl of 150 mM Tris–HCl buffer (pH 8.3) containing 1.125 M NaCl and 10 mM Abz-Gly-Phe-(NO_2)-Pro and preincubated 10 min at 37 °C. Finally the reaction mixture was 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 each collected fraction is expressed as percentage.

2.5. *Elucidation of the peptide sequence by tandem mass spectrometry*

Identification of the peptide sequence from fractions exhibiting remarkable ACE inhibitory activity was carried out by MS/MS analysis using an Ultimate Plus/Famos nano LC system (LC Packings, Amsterdam, The Netherlands) and a QSTAR XL hybrid quadrupole-TOF instrument (ABSCIEX, CA, USA) equipped with a nanoelectrospray ion source (Protana, Odense, Denmark). Fractions obtained from RP-HPLC fractionation showing the highest ACE inhibitory activity were concentrated using a speed-vac. Peptides were then desalted using C18 pipette tips and diluted in 60 μl of loading buffer (0.1% of formic acid and 2% of acetonitrile in water). Diluted samples were pre-concentrated on a 0.3 × 5 mm, 3 μm, C18 trap column from LC Packings PepMap (Dionex Company, Amsterdam, The Netherlands) at a flow rate of 40 μl/min and using 0.1% of trifluoroacetic acid (TFA) as mobile phase. After 3 min of pre-concentration, the trap column was automatically switched in-line with a 0.075 × 150 mm, 3 μm, Dionex C18 PepMap column from LC Packings. Mobile phases consisted of solvent A, containing 0.1% formic acid in water and solvent B containing 0.1% formic acid in 95% acetonitrile. Chromatographic conditions were a linear gradient from 95% to 50% solvent A in 30 min at a flow rate of 0.2 ml/min. The column outlet was directly coupled to a nano-eletrospray ion source (Protana, Odense, Denmark) using a 15 μm PicoTip EMITTER SilicaTip needle (New Objective, Massachusetts, USA). The positive TOF mass spectra were recorded on the QSTAR instrument using information dependent acquisition (IDA). TOF MS survey scan was recorded for mass range m/z 350–1800 followed by MS/MS scans of the three most intense peaks. Typical ion spray voltage was in the range of 2.5–3.0 kV, and nitrogen was used as collision gas. Other source parameters and spray position were optimized with a tryptic digest of protein mixture digest (LC Packings; P/N 161088). Interpretation of obtained MS/MS spectra was performed using an in-house 2.3 Mascot search engine version (Matrix Science, London, England) in combination with searches on UniProtKB/Swiss-Prot protein database. Mascot.dll 1.6b25 and ABSciex.DataAccess.Wiff File DataReader.dll were used for importing data to Mascot. Searches were done with the "none" enzyme specificity and a tolerance on the mass measurement of 0.7 Da in MS mode and 0.5 Da for MS/MS ions. Deamidation of Asn-Gln and oxidation of Met were used as variable modifications.

2.6. *Statistical analysis*

Each experiment was performed on two separate replications. One-way analysis of variance (ANOVA) was used (Minitab Statistic Program, release 8.21; Minitab Inc., Philadelphia, PA, USA).

3. Results and discussion

Cell counts in pork sarcoplasmic and myofibrillar meat protein extracts, both inoculated with *L. sakei* CRL1862 or *L. curvatus* CRL705 (WC + CFE), showed initial numbers of approximately 10^9 CFU/ml. After 96 h of incubation at 30 °C, a decline to 8.21 log CFU/ml and undetectable levels for *L. sakei* CRL1862 were found in sarcoplasmic and myofibrillar extracts respectively, while decreases to 7.41 (sarcoplasmic) and 2.39 (myofibrillar) log CFU/ml were observed for *L. curvatus* CRL705 (Table 1). These results are in agreement with those previously reported by Fadda et al. [15], in which *L. curvatus* CECT904 and *L. sakei* CECT4808 (WC + CFE) incubated on sarcoplasmic proteins under similar conditions yielded higher final viabilities than myofibrillar extracts. On the other hand, the combined WC and CFE suspensions inoculated on myofibrillar extracts in this study exhibited the same trend as was reported by Sanz et al. [14].

L. sakei CRL1862 and *L. curvatus* CRL705 were reported to exert antimicrobial activity [18,19]. With the aim to use these strains as potential functional cultures in meat products, the evaluation of their protein hydrolytic ability as well as bioactive peptides generation (in particular ACE inhibitory activity) from pork sarcoplasmic and myofibrillar proteins was carried out. A biochemical approach is presented in this work using proteolytic enzymes available in both WC and CFE suspensions with the objective to enhance the proteolytic activity on meat proteins. In fact, CFE from different microorganisms were applied as an additional enzyme source to achieve higher availability of small peptides and free amino acids in dry fermented sausages [20].

The resulting peptide patterns obtained from the proteolytic activity of *L. sakei* CRL1862 and *L. curvatus* CRL705 on sarcoplasmic proteins are shown in Fig. 1. The RP-HPLC profiles from these extracts showed minor differences between 0 and 96 h in control samples (Fig. 1A,B) when compared to those inoculated with the combination of WC and CFE from *L. sakei* CRL1862 (Fig. 1C) and *L. curvatus* CRL705 (Fig. 1D). Indeed, inoculated extracts showed great peptide generation between 15 and 40 min and the disappearance of

Table 1 – Cell count (±SD) in meat extracts incubated at 30 °C for 96 h.

	Cells count (CFU/ml) ± SD			
	Sarcoplasmic		Myofibrillar	
	0	96	0	96
L. sakei CRL1862	9.72 ± 0.19a	8.21 ± 0.15a	9.37 ± 0.18a	ND
L. curvatus CRL705	9.50 ± 0.16a	7.41 ± 0.18b	9.24 ± 0.14a	2.39 ± 0.17c
Control	ND	ND	ND	ND

Means with different letters in the same column differ significantly ($P < 0.05$); ND: not detectable.

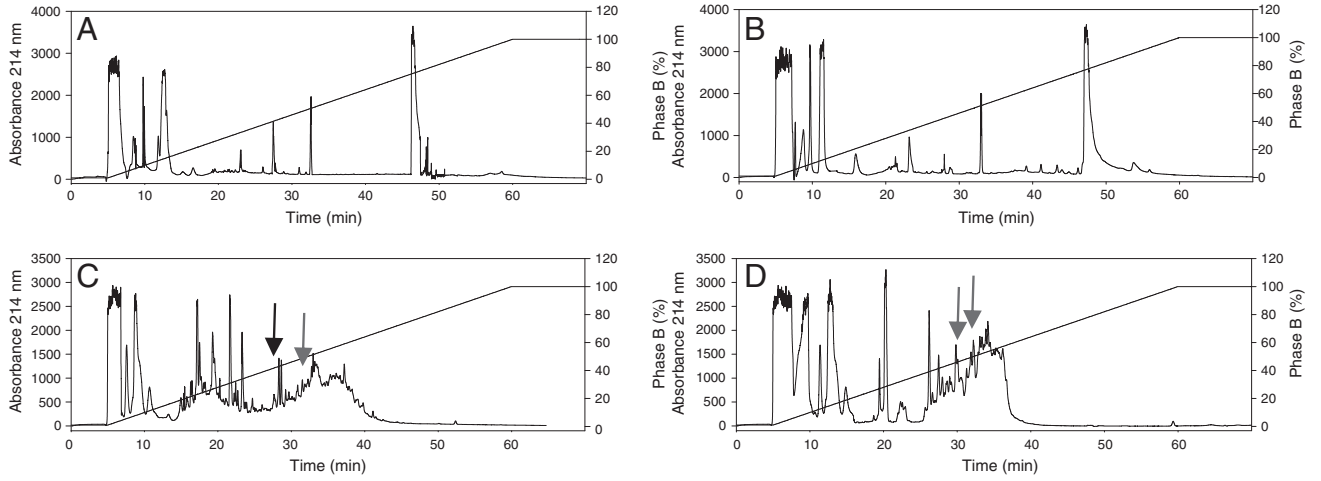

Fig. 1 – RP-HPLC separation of fractions from sarcoplasmic protein extract. Control samples at 0 (A) and 96 h (B) of incubation; samples inoculated with *L. sakei* CRL1862 (WC + CFE) at 96 h (C); samples inoculated with *L. curvatus* CRL705 (WC + CFE) at 96 h (D) of incubation at 30 °C. Arrows indicate active fractions. Chromatography conditions were performed as described in the Materials and methods.

peaks after 45 min in contrast to control samples at 96 h of incubation. It has been reported that peptides rich in hydrophobic amino acids and present in the medium–high retention time region of a chromatogram on C18 reverse phase column may exhibit ACE inhibitory activity [21]. Myofibrillar extracts showed similar results than those for sarcoplasmic protein extracts, since again no significant changes were found between myofibrillar control samples at times 0 and 96 h (Fig. 2A,B). During 96 h of incubation with the addition of WC and CFE from *L. sakei* CRL1862 and *L. curvatus* CRL705, some new peaks were generated by both organisms like those at 12, 19 and 28 min of retention time (Fig. 2C,D). However, the generation of peptides from sarcoplasmic proteins was much higher than that from myofibrillar. In coincidence with these results, lactobacilli strains were reported to hydrolyze pork sarcoplasmic proteins while a lack of proteolytic activity was detected on myofibrillar extracts [22]. In contrast, an intense hydrolysis of myofibrillar proteins during fermentation and drying stages of pork sausages using a mixed starter culture (LAB and *Staphylococcus* strains) was found by Aro Aro et al. [23] and Hughes et al. [24].

When the ACE inhibitory activity was determined, only the hydrolysates derived from sarcoplasmic proteins by the action of *L. sakei* CRL1862 and *L. curvatus* CRL705 resulted to be active. Peptides generated by *L. sakei* CRL1862 from sarcoplasmic extracts after 96 h of incubation showed inhibitory activities toward ACE of 54% ± 2.3 and 50% ± 3.1 in 27 and 32 min fractions, respectively (Fig. 1C). Also high percentages

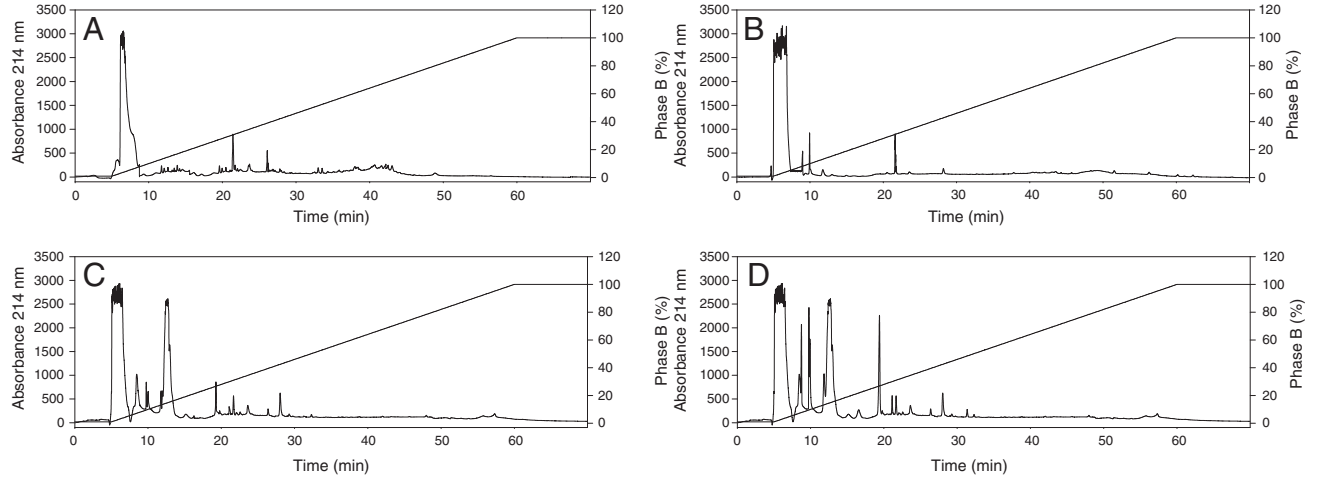

Fig. 2 – RP-HPLC separation patterns of soluble peptides contained in myofibrillar protein extract. Control samples at 0 (A) and 96 h (B) of incubation; samples inoculated with *L. sakei* CRL1862 (WC + CFE) at 96 h (C); samples inoculated with *L. curvatus* CRL705 (WC + CFE) at 96 h (D) of incubation at 30 °C. Chromatography conditions were performed as described in the Materials and methods.

of inhibition (48% ± 3.4 and 50% ± 2.8) were detected in the fractions eluting at 32 and 34 min from *L. curvatus* CRL705 hydrolysates (Fig. 1D). The remaining collected fractions did not exert a remarkable ACE inhibition. Similar ACE inhibitory activities from sarcoplasmic proteins isolated from bovine muscles were found using thermolysin + proteinase A and papain enzymes [25,26]. In addition, when *L. fermentum* SM 605 was used as enzyme source on marine proteins, high ACE inhibitory activity particularly during fermentation of marine shrimp (*A. chinensis*) was obtained [11].

The identification of peptides contained in fractions 27 and 32 (Fig. 1C) and fractions 32 and 34 (Fig. 1D) was carried out through MS/MS ion search using the Mascot search engine and UniProtKB/Swiss-Prot protein database. Results from peptide sequencing were subjected to a manual evaluation as described by Chen et al. [27]; the validated sequences explained all the major peaks found in the full MS spectrum. The active ACE inhibitory fractions containing one or more peptides, their respective molecular masses and sequences, are listed in Tables 2 and 3. A search of potential biological activity that can be derived from the identified peptides was carried out with the BioPep database and by comparison with sequences previously reported [28,29]. Fraction 27, obtained from sarcoplasmic proteins by the action of *L. sakei* CRL1862 showed the highest ACE inhibition activity (54%), yielding only one peptide sequence (FISNHAY), whereas a total of 17 peptides were identified from fraction 32 of the same extract. On the other hand, 50 peptides were characterized from the two most active fractions originated by *L. curvatus* CRL705, 30 from fraction 32 and 20 from fraction 34 (Table 3). Indeed, both fractions corresponding to a retention time of 32 min were found to be coincident for LAB strains in terms of ACE inhibitory activity; however, as shown in Tables 2 and 3, the nature of peptides contained in them differs significantly.

In general, all the peptides identified from the 4 analyzed fractions showed a prevalence of hydrophobic and neutral residues in the penultimate and ultimate C-terminal position. It has been reported that at penultimate C-terminal position, aliphatic (V, I, and A), basic (R) and aromatic (Y and F) residues are frequently found in ACE inhibitory peptides, while aromatic (W, Y, and F), proline (P) and aliphatic (I, A, L and M) residues are preferred at the ultimate C-terminal end of peptides [30]. In addition, peptides consisting of 6–14 amino acid residues were identified (Tables 2 and 3), which are in coincidence with ACE inhibitory peptides containing this numbers of residues reported by Byun and Kim [31]. Although small peptides (tri or tetra) were described to be more easily absorbed in the intestinal tract than larger peptides [32], evidence that large peptides could also be absorbed through the small intestine wall of rats was previously reported [33]. In this study, *L. sakei* CRL1862 as shown in Table 3 was able to generate the unique peptide FISNHAY in fraction 27. The heptapeptide FISNHAY, rather than having a proline residue, has aliphatic (alanine) and aromatic (tyrosine) residues at the penultimate and ultimate C-terminal positions, respectively; this peptide sequence has not yet been found in other protein

Table 2 – Bioactive peptides identified from pork sarcoplasmic proteins by the hydrolytic action of *L. sakei* CRL1862 during 96 h at 30 °C.

Fraction	Obs mass (Da) charge state	Calc mass	Identified peptide containing the sequence	Protein origin (UniProtKB/Swiss-Prot protein entry name)	ACE inhibitory sequences reported in the literature [a]
27	426.211^{2+}	850.4	FISNHAY	Fructose-bisphosphate aldolase (*Bos taurus*)	AY
32	377.2^{2+}	752.4	AMQKIF oxidation (M) [b]	Beta-enolase (ENOB_BOVIN)	IF,QF
	631.36^{2+}	1891.05	LKTAIQAAGYPDKV oxidation (M); deamidated (NQ) [c]	Beta-enolase (ENOB_BOVIN)	GY,YP,AA,AG,AI
	638.85^{2+}	1275.68	IKNYPVVSIED	Beta-enolase (ENOB_BOVIN)	YP,NY,IE
	496.59^{2+}	1486.77	AAVYKALSDHHIY	Fructose-bisphosphate aldolase (ALDOA_RABIT)	IY,VY,AA,YK,KA
	442.72^{2+}	883.43	SDHHIYL	Fructose-bisphosphate aldolase (ALDOA_RABIT)	IY,YL
	717.35^{2+}	1432.69	LSGGQSEEEASINL	Fructose-bisphosphate aldolase (ALDOA_RABIT)	GQ,GG,SG,EA
	557.3^{2+}	1112.58	TFSYGRALQA	Fructose-bisphosphate aldolase (ALDOA_RABIT)	YG,RA,GR,SY,LQ,TF
	426.211^{2+}	850.4	FISNHAY	Fructose-bisphosphate aldolase (ALDOA_RABIT)	AY
	669.43^{1+}		MVLPPP	Thrombospondin type-1 domain-containing protein 1 (THSD1_MOUSE)	VLP,LPP,PP
	440.74^{2+}	879.48	VGLDTTKF	Hydroxyacyl-coenzyme A dehydrogenase (HCDH_PIG)	VG,GL,KF
	502.78^{2+}	1003.55	VGGASLKPEF	Triosephosphate isomerase (TPIS_BOVIN)	LKP,VG,GA,GG,KP
	430.26^{2+}	858.5	SPLPVIPH	LIM domain-binding protein 3 (LDB3_MOUSE)	PLP,PL,IP,PH
	435.77^{2+}	869.53	MPQQIGVP	Serine/threonine-protein kinase (WNK1_MOUSE)	IG,GV,PQ
	547.78^{2+}	1093.54	KETPSGFTLD	Creatine kinase M-type (KCRM_BOVIN)	GF,SG,KE
	731.4^{1+}		VIQTGVD	Creatine kinase M-type (KCRM_BOVIN)	GV,TG
	542.29^{2+}	1082.58	YYKATEPVI	Adenylate kinase isoenzyme (KAD1_BOVIN)	YK,KA,TE
	388.23^{2+}	774.44	PAKIEAF		AF,EA,IE

[a] BioPep database.
[b] Oxidation.
[c] Deamidation.

Table 3 – Bioactive peptides identified from pork sarcoplasmic proteins by the hydrolytic action of *L. curvatus* CRL705 during 96 h at 30 °C.

Fraction	Obs mass (Da) charge state	Calc mass	Identified peptide containing the sequence	Parent protein	ACE inhibitory sequence reported in the literature [a]
32	502.78^{2+}	1003.55	VGGASLKPEF	Triosephosphate isomerase (TPIS_BOVIN)	LKP,VG,GA,GG,KP
	440.75^{2+}	879.48	VGLDTTKF	Hydroxyacyl-coenzyme A dehydrogenase (HCDH_PIG)	VG,GL,KF
	500.78^{2+}	999.55	EVGGEALGRL	Hemoglobin subunit beta (HBB_CALTO)	VG,GR,GE,GG,LG,EA,EV
	417.27^{2+}	832.53	QLGKAGIM	Neurochondrin (NCDN_BOVIN)	GI,AG,GK,LG,KA
	615.34^{1+}		ASDPIL	Pyruvate kinase isozymes M1/M2 (KPYM_RAT)	
	601.34^{2+}	1200.66	ASDPILYRPVA	Pyruvate kinase isozymes M1/M2 (KPYM_RAT)	LY,RP
	464.79^{2+}	927.57	PILYRPVA	Pyruvate kinase isozymes M1/M2 (KPYM_RAT)	LY,RP
	515.73^{2+}	1029.46	SKHPGDFGAD	Myoglobin (MYG_PIG)	GA,FG,GD,PG,HP
	669.42^{1+}		IVPIVE	Fructose-bisphosphate aldolase A (ALDOA_RABIT)	VP,VE
	496.6^{3+}	1486.78	AAVYKALSDHHIY	Fructose-bisphosphate aldolase A (ALDOA_RABIT)	IY,VY,AA,YK,KA
	717.37^{2+}	1432.72	LSGGQSEEEASINL	Fructose-bisphosphate aldolase A (ALDOA_RABIT)	GQ,GG,SG,EA,
	426.211^{2+}	850.4	FISNHAY	Fructose-bisphosphate aldolase A (ALDOA_RABIT)	AY
	430.26^{2+}	858.5	SPLPVIPH	LIM domain-binding protein 3 (LDB3_MOUSE)	PLP,PL,IP,PH
	430.27^{2+}	1287.8	KLDVKGKRVVM	Phosphoglycerate kinase 1 (PGK1_BOVIN)	VK,KR,KG,GK,KL
	464.72^{2+}	927.43	MSHLGRPD	Phosphoglycerate kinase 1 (PGK1_BOVIN)	GRP,RP,HL,GR,LG
	541.28^{2+}	1080.54	IKWGDAGATY	Glyceraldehyde-3-phosphate dehydrogenase (G3P_PIG)	KW,GA,AG,DA,WG,GD
	654.28		GDAGATY	Glyceraldehyde-3-phosphate dehydrogenase (G3P_PIG)	GA,AG,DA,GD
	837.44		VVESTGVF	Glyceraldehyde-3-phosphate dehydrogenase (G3P_PIG)	VF,GV,TG,VE
	511.77^{2+}	1021.53	GYSNRVVDL	Glyceraldehyde-3-phosphate dehydrogenase (G3P_PIG)	GY
	377.2^{2+}	752.4	AMQKIF	Beta-enolase (ENOB_BOVIN)	IF,QK
	594.79^{2+}	1187.57	DSRGNPTVEVD	Beta-enolase (ENOB_BOVIN)	EV,VE,PT
	331.7^{2+}	661.39	KVVIGM	Beta-enolase (ENOB_BOVIN)	IG,GM
	534.3		VVIGM	Beta-enolase (ENOB_BOVIN)	IG,GM
	638.85^{2+}	1275.69	IKNYPVVSIED	Beta-enolase (ENOB_BOVIN)	YP,NY,IE
	519.3^{2+}	1036.59	NTNHGRILL	Glucose-6-phosphate isomerase (G6PI_PIG)	GR,HG
	420.75^{2+}	839.49	GTHIAKTL	Glucose-6-phosphate isomerase (G6PI_PIG)	IA,GT,IAK
	513.79^{2+}	1025.38	PSAVAKHFVA	Glucose-6-phosphate isomerase (G6PI_PIG)	
	731.4		VIQTGVD	Creatine kinase M-type	GV,TG
	508.28		VGSVF	Creatine kinase M-type	VF,VG,GS
	435.77^{2+}	869.53	GGKDQRLP	Membrane metallo-endopeptidase-like 1 (MMEL1_MOUSE)	RL,GK,GG
34	559.33	1116.64	LVGGASLKPEF	Triosephosphate isomerase (TPIS_BOVIN)	LKP,VG,GA,GG,KP
	323.22^{2+}	644.43	KSLLGK	Phosphoglycerate kinase 1 (PGK1_BOVIN)	GK,LG
	380.74^{2+}	759.46	KSLLGKD	Phosphoglycerate kinase 1 (PGK1_BOVIN)	GK,LG
	486.82^{2+}	971.62	KSLLGKDVL	Phosphoglycerate kinase 1 (PGK1_BOVIN)	GK,LG
	649.39^{2+}	1296.78	LEGKVLPGVDALS	Phosphoglycerate kinase 1 (PGK1_BOVIN)	VLP,LPG,DA,GV,GK,EG,PG
	527.28^{3+}	1578.83	PFGNTHNKYKLNF	Creatine kinase M-type (KCRM_PIG)	FG,NF,KY,YK,NK,LN
	407.71^{2+}	813.41	PGHPFIM	Creatine kinase M-type (KCRM_PIG)	GH,PG,HP
	380.2^{2+}	758.38	IDDHFL	Creatine kinase M-type (KCRM_PIG)	
	377.25^{2+}	752.49	KPVSPLL	Creatine kinase M-type (KCRM_PIG)	VSP,PL,KP
	751.42		TAAVGSVF	Creatine kinase M-type (KCRM_PIG)	VF,AA,VG,GS
	495.29^{2+}	988.57	LVVDGVKLM (M) [b]	Creatine kinase M-type (KCRM_PIG)	VK,GV,DG,KL
	475.29^{2+}	948.56	YVTAIRNL	Serotransferrin (TRFE_HORSE)	IR,AI
	314.7^{2+}	627.39	VILFH	Fructose-bisphosphate aldolase A (ALDOA_RABIT)	LF
	534.3^{3+}	1599.88	AAVYKALSDHHIYL	Fructose-bisphosphate aldolase A (ALDOA_RABIT)	IY,VY,YL,AA,YK,KA
	453.92^{3+}	1358.74	YKALSDHHIYL	Fructose-bisphosphate aldolase A (ALDOA_RABIT)	IY,YL,YK,KA
	483.25^{2+}	964.49	FISNHAY (NQ) [c]	Fructose-bisphosphate aldolase A (ALDOA_RABIT)	AY
	547.8^{2+}	1093.59	VDTSKGFLID	Adenylate kinase isoenzyme (KAD1_BOVIN)	GF,KG
	549.85^{2+}	1097.68	PILYRPVAVA	Pyruvate kinase isozymes (KPYM_RABIT)	LY,RP,VAV

Table 3 (*continued*)

Fraction	Obs mass (Da) charge state	Calc mass	Identified peptide containing the sequence	Parent protein	ACE inhibitory sequence reported in the literature [a]
34	478.26^{3+}	1431.76	QQAVRAIVSCFPN	Beta-1,3-galactosyl-O-glycosyl-glycoprotein beta-1,6-N-acetylglucosaminyltransferase (GCNT3_RAT)	FP,RA,VR,CF,AI
	545.83^{2+}	1089.64	QLVMFVLQL	Anion exchange transporter (S26A7_MOUSE)	MF,LQ

[a] BioPep database.
[b] Oxidation.
[c] Deamidation.

hydrolysates with ACE inhibitory activity. In addition, the peptide VGGASLKPEF generated by both *L. sakei* CRL1862 and *L. curvatus* CRL705 (fraction 32) and the peptide LVGGASLKPEF generated by *L. curvatus* CRL705 in fraction 34 showed the bioactive tripeptide LKP [30] encrypted in their sequences. Similarly, this bioactive sequence was also found in the peptide LKPNM derived from dried bonito muscle digested with thermolysin, which has been reported to exhibit ACE inhibitory activity [34]. In particular, when fractions generated by *L. curvatus* CRL705 from sarcoplasmic proteins were analyzed, the peptides PILYRPVA and VVIGM identified from fraction 32 matched as part of the longer peptides ASDPILYRPVA and KVVIGM, respectively, which were also found in this fraction. Moreover, the peptides originated from phosphoglycerate kinase, KSLLGKD and KSLLGKDVL differing in one and two amino acid, respectively and the peptide KSLLGK which were found to share a strong homology were identified from the fraction 34. Notably, ACE inhibitory activity was similar in all four active fractions containing the peptide FISNHAY. Because this peptide was the only identified from the fraction 27, the observed inhibitory activity in the remaining fractions may be attributed to this peptide. Moreover the large amount of peptides generated from sarcoplasmic extracts confirm the existence of remarkable exopeptidase activities in the cell suspensions of *L. sakei* CRL1862 and *L. curvatus* CRL705, as was previously pointed out [35,19].

4. Conclusions

This study demonstrated that antihypertensive activity can be generated from porcine proteins fermentation by using lactic acid bacteria, through the release of ACE inhibitory peptides. The generated bioactive peptides by *L. sakei* CRL1862 and *L. curvatus* CRL705 may be used to develop physiologically functional foods, giving rise to new market for healthier meat products. In this way, further studies must be carried out in order to evaluate in vivo the antihypertensive activity of the hydrolysates.

Acknowledgments

Grant AGL2010-16305 from MCyT (Madrid, Spain) and FEDER funds are acknowledged. P. Castellano received a postdoctoral fellowship from the Consejo Nacional de Investigaciones Cientificas y Técnicas (CONICET), Argentina.

REFERENCES

[1] Friedman M. Nutritional value of proteins from different food sources: a review. J Agric Food Chem 1996;44:6–29.
[2] Korhonen H, Pihlanto A. Bioactive peptides: production and functionality. Int Dairy J 2006;16:945–60.
[3] Sikorski ZE. The role of proteins in food. In: Sikorski ZE, editor. Chemical and functional properties of food components. 3rd ed. Boca Raton: CRC Press; 2006. p. 129–75.
[4] Udenigwe CC, Aluko RE. Food protein-derived bioactive peptides: production, processing, and potential health benefits. J Food Sci 2012;71:R11–24.
[5] Erdös EG, Tan F, Skidgel RA. Angiotensin I-converting enzyme inhibitors are allosteric enhancers of kinin B1 and B2 receptor function. Hypertension 2010;55:214–20.
[6] De Leo F, Panarese S, Gallerani R, Ceci LR. Angiotensin converting enzyme (ACE) inhibitory peptides: production and implementation of functional food. Curr Pharm Des 2009;15:3622–43.
[7] Ryan JT, Ross RP, Bolton D, Fitzgerald GF, Stanton C. Bioactive peptides from muscle sources: meat and fish. Nutrients 2011;3:765–91.
[8] Gobbetti M, Stepaniak L, Angelis MD, Corsetti A, Cagno RD. Latent bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing. Crit Rev Food Sci Nutr 2002;42:223–39.
[9] Minervini F, Algaron F, Rizzello CG, Fox PF, Monet V, Gobbetti M. Angiotensin I-converting-enzyme-inhibitory and antibacterial peptides from *Lactobacillus helveticus* PR4 proteinase hydrolyzed caseins of milk from six species. Appl Environ Microbiol 2003;69:5297–305.
[10] Rodríguez-Figueroa JC, González-Córdova AF, Torres-Llanez MJ, Garcia HS, Vallejo-Cordoba B. Novel angiotensin I-converting enzyme inhibitory peptides produced in fermented milk by specific wild *Lactococcus lactis* strains. J Dairy Sci 2012;95:5536–43.
[11] Wang YK, He HL, Chen XL, Sun CY, Zhang YZ, Zhou BC. Production of novel angiotensin I-converting enzyme inhibitory peptides by fermentation of marine shrimp *Acetes chinensis* with *Lactobacillus fermentum* SM 605. Appl Microbiol Biotechnol 2008;79:785–91.
[12] Vignolo GM, Suriani F, Pesce de Ruiz Holgado A, Oliver G. Antibacterial activity of *Lactobacillus* strains isolated from dry fermented sausages. J Appl Bacteriol 1993;75:344–9.
[13] Castro MP, Palavecino NZ, Herman C, Garro OA, Campos CA. Lactic acid bacteria isolated from artisanal dry sausages: characterization of antibacterial compounds and study of the factors affecting bacteriocin production. Meat Sci 2011;87:321–9.
[14] Sanz Y, Fadda S, Vignolo G, Aristoy MC, Oliver G, Toldrá F. Hydrolysis of muscle myofibrillar proteins by *Lactobacillus curvatus* and *Lactobacillus sake*. Int J Food Microbiol 1999;53:115–25.

[15] Fadda S, Sanz Y, Vignolo G, Aristoy MC, Oliver G, Toldrá F. Characterization of muscle sarcoplasmic and myofibrillar proteins hydrolysis caused by *Lactobacillus plantarum*. Appl Environ Microbiol 1999;65:3540–6.
[16] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
[17] Sentandreu MA, Toldrá F. A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme. Food Chem 2006;97:546–54.
[18] Castellano P, Vignolo G. Inhibition of *Listeria innocua* and *Brochothrix thermosphacta* in vacuum-packaged meat by addition of bacteriocinogenic *Lactobacillus curvatus* CRL705 and its bacteriocins. Lett Appl Microbiol 2006;43:194–9.
[19] Castellano P, Aristoy MC, Sentandreu MA, Vignolo G, Toldrá F. *Lactobacillus sakei* CRL1862 improves safety and protein hydrolysis in meat systems. J Appl Microbiol 2012;113:1407–16.
[20] Flores M, Toldrá F. Microbial enzymatic activities for improved fermented meats. Trends Food Sci Technol 2011;22:81–90.
[21] Megias C, Yust MD, Pedroche J, Lquari H, Giron-Calle J, Alaiz M, et al. Purification of an ACE inhibitory peptide after hydrolysis of sunflower (*Helianthus annuus* L.) protein isolates. J Agric Food Chem 2004;52:1928–32.
[22] Villani F, Casaburi A, Pennacchia C, Filosa L, Russo F, Ercolini D. Microbial ecology of the soppressata of Vallo di Diano, a traditional dry fermented sausage from southern Italy, and in vitro and in situ selection of autochthonous starter cultures. Appl Environ Microbiol 2007;73:5453–63.
[23] Aro Aro JM, Nyam-Osor P, Tsuji K, Shimada K, Fukushima M, Sekikawa M. The effect of starter cultures on proteolytic changes and amino acid content in fermented sausages. Food Chem 2011;119:279–85.
[24] Hughes MC, Kerryb JP, Arendtb EK, Kenneallyc PM, McSweeneya PLH, O'Neilla EE. Characterization of proteolysis during the ripening of semi-dry fermented sausages. Meat Sci 2002;62:205–16.
[25] Jang A, Lee M. Purification and identification of angiotensin converting enzyme inhibitory peptides from beef hydrolysates. Meat Sci 2005;69:653–61.
[26] Di Bernardini R, Mullen AM, Bolton D, Kerry J, O'Neill E, Hayes M. Assessment of the angiotensin-I-converting enzyme (ACE-I) inhibitory and antioxidant activities of hydrolysates of bovine brisket sarcoplasmic proteins produced by papain and characterisation of associated bioactive peptidic fractions. Meat Sci 2012;90:226–35.
[27] Chen Y, Know SW, Kim SC, Zhao Y. Integrated approach for manual evaluation of peptides identified by searching protein sequence databases with tandem mass spectra. J Proteome Res 2005;4:998–1005.
[28] Vercruysse L, van Camp J, Smagghe G. ACE inhibitory peptides derived from enzymatic hydrolysates of animal muscle protein: A review. J Agric Food Chem 2005;53:8106–15.
[29] Escudero E, Sentandreu MA, Arikara K, Toldrá F. Angiotensin I-converting enzyme inhibitory peptides generated from in vitro gastrointestinal digestion of pork meat. J Agric Food Chem 2012;58:2895–901.
[30] Fujita H, Yokoyama K, Yoshikawa M. Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. J Food Sci 2000;65:564–9.
[31] Byun HG, Kim SK. Structure and activity of angiotensin I converting enzyme inhibitory peptides derived from Alaskan Pollack skin. J Biochem Mol Biol 2002;35:239–43.
[32] Hara H, Funabiki R, Iwata M, Yamazaki K. Portal absorption of small peptides in rats under unrestrained condition. J Nutr 1984;114:1122–9.
[33] Roberts PR, Burney JD, Black KW, Zaloga GP. Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. Digestion 1999;60:332–7.
[34] Curtis JM, Dennis D, Waddell DS, MacGillivray T, Ewart HS. Determination of angiotensin-converting enzyme inhibitory peptide Leu-Lys-Pro-Asn-Met (LKPNM) in bonito muscle hydrolysates by LC–MS/MS. J Agric Food Chem 2002;50: 3919–25.
[35] Sanz Y, Fadda S, Vignolo G, Aristoy MC, Oliver G, Toldrá F. Hydrolytic action of *Lactobacillus casei* CRL 705 on pork muscle sarcoplasmic and myofibrillar proteins. J Agric Food Chem 1999;47:3441–8.