Angiotensin-Converting Enzyme Inhibitory Activity of Peptides Derived from Caprine Kefir

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ABSTRACT

In this study, a potent angiotensin-converting enzyme (ACE)-inhibitory activity was found in a commercial kefir made from caprine milk. The low molecular mass peptides released from caseins during fermentation were mainly responsible for this activity. Sixteen peptides were identified by HPLC-tandem mass spectrometry. Two of these peptides, with sequences PYVRYL and LVYPFTGPIPN, showed potent ACE-inhibitory properties. The impact of gastrointestinal digestion on ACE-inhibitory activity of kefir peptides was also evaluated. Some of these peptides were resistant to the incubation with pepsin followed by hydrolysis with Corolase PP. The ACE-inhibitory activity after simulated digestion was similar to or slightly lower than unhydrolyzed peptides, except for peptide β -casein f(47-52) (DKIHPPF), which exhibited an activity 8 times greater after hydrolysis.

(Key words: angiotensin-converting enzyme-inhibitory activity, caprine kefir, simulated gastrointestinal digestion, mass spectrometry)

Abbreviation key: ACE = angiotensin-converting enzyme, IC₅₀ = protein concentration needed to inhibit the original ACE activity by 50%, MS/MS = tandem mass spectrometry, RP-HPLC = reverse phase-HPLC, WSE = water-soluble extract.

INTRODUCTION

Kefir is an ancient fermented milk beverage native to the Caucasian Mountains that has an exotic sour and slightly alcoholic flavor. This beverage is different from other fermented milks because it is the result of fermentation with a mixed microflora confined to a matrix of discrete “kefir grains” (Marshall et al., 1984). The microbial composition of the kefir grains depends on several factors, such as their origin, the cultivation and manufacturing methods, and the assay of microbial identification (Corli Witthuhn et al., 2004). Lactic acid

bacteria in the kefir grains have been reported to include homo- and heterofermentative *Lactobacillus*, *Lactococcus*, *Leuconostoc*, and some *Acetobacter*, and the yeasts include members of the genera *Saccharomyces*, *Candida*, *Kluyveromyces*, *Torulopsis*, and *Cryptococcus* (Simova et al., 2002). The lactic acid bacteria, yeast, and polysaccharide “kefiran” present in the kefir grains have been described as a symbiotic community that imparts unique properties to kefir (Beshkova et al., 2002). In fact, kefir, kefir grains, kefiran, and the bacteria found in kefir have been the subject of scientific studies to demonstrate their beneficial effects on animals and humans. Although historically kefir was prepared using the milk of sheep, goats, and cows (Wszolek et al., 2001), the interest of these studies has centered mainly on the biological activities of bovine kefir.

It is known that kefir has numerous biological activities including antitumor and immunostimulating effects in animals, an antioxidant action by reducing the lipid peroxidation, an antidiabetes effect, antibacterial and antifungal properties, and a probiotic or prebiotic effect (Farnworth and Mainville, 2003). Several studies in humans have shown that the consumption of kefir can reduce serum cholesterol levels (Vujicic et al., 1992), and can act as an alternative to yogurt for improving lactose digestion (Hertzler and Clancy, 2003). The information about the wholesome properties and the high nutritional value of kefir and the consideration of health and safety trends in recent years have increased the interest of consumers and manufacturers in this novel milk food.

Angiotensin-converting enzyme (ACE) is one of the main molecules responsible for controlling the blood pressure due to its action in the formation of angiotensin II, a potent vasoconstrictor, and in the degradation of bradykinin, a vasodilator. Thus, inhibition of ACE results in a lowering of blood pressure. In vitro ACE-inhibitory activity and in vivo antihypertensive effects have been reported for different fermented milks cultured in the laboratory with different strains of lactic acid bacteria (Gobbetti et al., 2000; Algaron et al., 2004; Ashar and Chand, 2004) and in commercial fermented milks (Hernández-Ledesma et al., 2004a). Moreover, the combined action of *Lactobacillus helveticus* and *Sac-*

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charomyces cerevisiae during the manufacture of bovine Calpis milk (Calpis Co. Ltd., Tokyo, Japan) resulted in the release from caseins of peptides VPP and IPP, which have been described to have potent ACE-inhibitory and antihypertensive activities (Nakamura et al., 1995; Takano, 1998). A low ACE-inhibitory activity but a high antihypertensive effect in spontaneously hypertensive rats was found in fermented milk cultured by various lactic acid bacteria and a yeast (Kuwabara et al., 1995). However, to date, there are no data available about the ACE-inhibitory activity of peptides released from caseins during kefir manufacture.

The aim of our study was to isolate and identify the ACE-inhibitory sequences included in the 3-kDa permeate obtained from a commercial caprine kefir. Furthermore, the behavior of these peptides after a 2-stage hydrolysis process (which simulates gastrointestinal digestion) was studied to evaluate both the survival and new formation of ACE-inhibitory peptides. The results of this work would be a preliminary step to predict the potential antihypertensive effect in vivo of this fermented milk.

MATERIALS AND METHODS

Preparation of Sample Extract

Four different batches of caprine kefir were purchased on the Spanish market throughout the year. As stated by the manufacturer, kefir was prepared using pasteurized milk cultured with kefir grains that contained the lactic acid bacteria *Lactococcus lactis*, *Lactococcus cremoris*, *Lactococcus* biovar. *diacetylactis*, *Leuconostoc mesenteroides* ssp. *cremoris*, *Lactobacillus plantarum*, *Lactobacillus casei*, and the yeast *Kluyveromyces marxianus* var. *fragilis* (*Torula kefir*).

Water-soluble extract (WSE) was obtained by centrifugation at $12,000 \times g$ for 10 min at 5°C and by filtration through Whatman no. 40 filter. The WSE was ultrafiltered using a regenerated cellulose membrane (3-kDa cut-off, $4.5 \times 10^{-3} \text{ m}^2$ effective membrane area; Millipore Corporation, Bedford, MA) equipment with a stirred ultrafiltration cell (model 8400, Millipore), a mini reservoir (RC 800, Millipore), and a concentration selector valve (CDS10, Millipore). The WSE and 3-kDa permeate were freeze-dried and kept at -20°C until use.

Measurement of ACE-Inhibitory Activity

Angiotensin-converting enzyme inhibitory activity was measured by the spectrophotometric assay of Cushman and Cheung (1971), with some modifications. Briefly, 40 μL of each sample was added to 0.1 mL of 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl, and 5 mM hippuryl-histidyl-leucine (Sigma

Chemical, St. Louis, MO). Angiotensin-converting enzyme (EC 3.4.15.1, 2 mU, 5.1 U/mg; Sigma) was added and the reaction mixture was incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.15 mL of 1 M HCl. The hippuric acid formed was extracted with ethyl acetate, heat-evaporated at 95°C for 10 min, redissolved in distilled water, and measured spectrophotometrically at 228 nm. The activity of each sample was tested in triplicate.

The ACE-inhibitory activity was calculated as the protein concentration needed to inhibit 50% of the original ACE activity (IC_{50}). A nonlinear adjustment of the data obtained was performed to calculate the IC_{50} values with the program PRISM version 4.02 for Windows (GraphPad Software, Inc., San Diego, CA). This program calculates the estimated value of the IC_{50} together with the standard error. Protein content of the WSE and 3-kDa permeate was determined by the Kjeldahl method, and protein content of the peptidic fractions was estimated by the bicinchoninic acid method (Pierce, Rockford, IL), using BSA as standard protein.

Separation of ACE-Inhibitory Peptides by Reverse Phase-HPLC

Semipreparative reverse phase-HPLC (RP-HPLC) analysis of the 3-kDa permeate from caprine kefir was carried out on a Waters System HPLC (Waters Corp., Milford, MA), according to Hernández-Ledesma et al. (2004a). The sample concentration was approximately 150 mg/mL and the injection volume was 1200 μL . Solvent A was a mixture of water and trifluoroacetic acid (1000:1 vol/vol), and solvent B contained acetonitrile-trifluoroacetic acid (1000:0.8 vol/vol). The peptides were eluted with a linear gradient of solvent B in A going from 0 to 30% in 70 min. Eight fractions were collected from 12 to 14 separate RP-HPLC runs, pooled, dried under vacuum, and redissolved in distilled water. Protein concentration and ACE-inhibitory activity (IC_{50}) were tested for each fraction.

A new preparative RP-HPLC step of 3-kDa permeate was carried out to get a better elution of peptides in the most active fraction collected in the preliminary step described above. The chromatographic conditions were similar but the linear gradient used for solvent B in A went from 8 to 20% in 45 min. Sample concentrations were approximately 150 mg/mL and the injection volume was 1000 μL . Each chromatographic run was repeated 45 times and 5 new subfractions derived from the most potent fraction were pooled, frozen, and lyophilized. Protein concentration and ACE-inhibitory activity (IC_{50}) were tested for each subfraction.

Analysis by On-Line RP-HPLC Tandem Mass Spectrometry

Reverse phase-HPLC separations of the most active fractions and subfractions collected from 3-kDa permeate of caprine kefir and peptide identification were performed on an Agilent HPLC system connected on line to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany), according to Hernández-Ledesma et al. (2004b). The active fractions were fractionated using a gradient from 13 to 31% in 70 min, and the peptides included in the most active subfractions were eluted with a linear gradient of solvent B in A going from 8 to 20% in 45 min.

Tandem mass spectrometry (MS/MS) was carried out to determine the amino acid sequence of the peptides included in these RP-HPLC fractions. Mass spectra were acquired over the range 50 to 1500 m/z (depending on the m/z and the charge state of the precursor ion). The statistical treatment of the MS data was carried out with the program, giving the estimated value of the calculated mass together with the standard error.

Simulation of Gastrointestinal Digestion of ACE-Inhibitory Peptides

Angiotensin-converting enzyme inhibitory peptides were prepared by a conventional solid-phase synthesis method with a 431 A peptide synthesizer (Applied Biosystems Inc., Uberlingen, Germany).

Simulation of gastrointestinal digestion of synthetic ACE-inhibitory peptides was carried out according to the method of Alting et al. (1997), with some modifications. Briefly, peptides dissolved in water (0.7% wt/vol) were hydrolyzed with pepsin (EC 3.4.23.1; 1:10,000, 890 U/mg of protein, 20 mg/g of peptide; Sigma) for 90 min at 37°C at pH 2.5 followed by hydrolysis with Corolase PP (40 mg/g of peptide; Röhm, Darmstadt, Germany) at pH 7.5 and 37°C for 150 min. During hydrolysis, pH was continuously measured and held constant by addition of HCl and NaOH (1 M). Hydrolysates were centrifuged at 35,000 $\times g$ for 30 min and the supernatants were stored at -20°C until further analysis.

Angiotensin-converting enzyme inhibitory activity was measured in the peptide hydrolysates. Moreover, the peptides and their hydrolysates were subjected to HPLC-MS/MS using the same equipment as described above, but the gradient used for solvent B in A went from 0 to 45% in 60 min.

RESULTS AND DISCUSSION

ACE-Inhibitory Activity of Fractions from Caprine Kefir

ACE-inhibitory activity of the WSE from caprine kefir was measured and calculated as IC_{50} . The IC_{50} value

of WSE was 0.365 ± 0.029 mg/mL. The activity was higher than that reported by Leclerc et al. (2002) for whey fractions obtained from casein-enriched milk fermented by *Lactobacillus helveticus* (0.6 to 1.1 mg/mL). To date, the most potent ACE-inhibitory peptides reported were released from proteins of milk fermented with several isolated strains of *Lactobacillus helveticus* (Yamamoto et al., 1999; Sipola et al., 2002) or combined with yeast *Saccharomyces cerevisiae* (Nakamura et al., 1995). Thus, the higher ACE-inhibitory activity determined in kefir could be due to the combined action of several strains of lactic acid bacteria and yeast during milk fermentation.

The 3-kDa permeate was obtained from WSE and its ACE-inhibitory activity was measured. The activity (IC_{50} of 0.380 ± 0.023 mg/mL) was similar to that determined in the WSE and higher than that found in the retentate (1.25 ± 0.063 mg/mL). These results revealed that small peptides are mainly responsible for the ACE-inhibitory activity of caprine kefir WSE. Short peptides exhibiting an ACE-inhibitory effect have been isolated and characterized from fermented milks (Gobbetti et al., 2000) and cheeses (Saito et al., 2000; Gómez-Ruiz et al., 2002).

With the aim of identifying the ACE-inhibitory peptides contained in caprine kefir, the 3-kDa permeate was selected and subjected to RP-HPLC on a preparative scale, giving an absorbance profile as shown in Figure 1A. The total chromatogram was separated into 8 fractions, named F1 to F8. Enough material from each fraction was collected in successive analyses to determine the protein concentration and the ACE-inhibitory activity. Figure 1A shows the IC_{50} values calculated for 8 collected fractions. All fractions showed ACE-inhibitory activity, with IC_{50} values varying from 21.8 to 416.0 mg/mL. For all measurements, the standard error was lower than 10%. Higher ACE-inhibition was measured for fractions F5, F6, and F7, which had IC_{50} values of 21.8, 60.1, and 58.7 μ g/mL, respectively. These fractions were selected for further identification studies.

Reverse phase-HPLC analysis revealed that fraction F5 was too complex for direct peptide identification by HPLC-MS/MS. Because of this, a new chromatographic analysis of 3-kDa permeate was carried out to elute the peptides of F5 with a shallower acetonitrile gradient. Five subfractions (F5.1 to F5.5) were collected and tested for protein concentration and ACE-inhibitory activity (Figure 1B). The IC_{50} values of the first 3 eluted subfractions (F5.1 to F5.3) were in the 48.2 to 91.6 μ g/mL range. This ACE-inhibitory activity was lower than that found for the whole F5 fraction. However, a potent ACE-inhibitory effect was observed for subfractions F5.4 and F5.5, which had similar IC_{50} values (8.1 μ g/

Figure 1. A) Ultraviolet chromatogram obtained by preparative reverse phase (RP)-HPLC of the 3-kDa permeate from the caprine kefir water-soluble extract (F1 to F8 represent the 8 collected fractions) and angiotensin-converting enzyme (ACE)-inhibitory activity, expressed as IC_{50} (■) of the collected fractions from the preparative RP-HPLC system (1A). B) Ultraviolet chromatogram obtained by preparative RP-HPLC of the fraction F5 shown in Figure 1A, and ACE-inhibitory activity, expressed as IC_{50} (●), of the 5 collected subfractions (F5.1 to F5.5).

mL). The most potent subfractions were selected to identify the peptides responsible for ACE-inhibitory activity. The identification of ACE-inhibitory peptides was also carried out in fractions F6 and F7. For these fractions, a preliminary purification step by RP-HPLC was not required because their peptidic patterns were less complicated to analyze.

Identification of ACE-Inhibitory Peptides by RP-HPLC-MS/MS

With the aim of identifying the ACE-inhibitory peptides, subfractions F5.4 and F5.5 and fractions F6 and F7 were subjected to RP-HPLC coupled on line to a mass spectrometer. All peptides of the total ion chromatogram with a signal higher than 10,000 units were considered for peptide sequencing. Only a few detected masses and the corresponding fragmentation spectra obtained by MS/MS could not be matched with any peptide fragment originated by casein hydrolysis during milk fermentation. As an example, Figure 2A shows the mass spectrum of one chromatographic peak of sub-

fraction F5.4, and Figure 2B shows the MS/MS spectrum of a singly charged ion with m/z 698.3, the amino acid sequence of the identified peptide, and the major fragment ions. The most intense fragment ions corresponded to those identified as y_2 , b_2 , y_4 , and b_4 . Ions y_2 and b_4 resulted from cleavage of the peptide bond Val-Pro. It has recently been reported that the most abundant Xxx-Pro relative bond cleavage ratios were observed when Xxx was Val, His, Asp, Ile, or Leu (Breci et al., 2003). Moreover, the presence of Lys at the C-terminus of this peptide favored the appearance of the y -type fragment ions in this spectrum.

The search for the masses and partial sequences (sequence tags) was carried out using a database of caprine milk proteins, including sequence modifications due to genetic variants. Sixteen peptide fragments were identified in the 4 fractions analyzed following this methodology, of which 10 corresponded to β -CN fragments, 5 to α_{s1} - and α_{s2} -CN fragments, and 1 to a κ -CN fragment. It must be noted that any identified fragment was derived from whey proteins. This could be due to the lowest susceptibility of these proteins to the proteolytic

Figure 2. A) Mass spectrum of one chromatographic peak of subfraction F5.4 collected from the 3-kDa permeate of caprine kefir water-soluble extract. B) Tandem mass spectrometry (MS/MS) spectrum of ion m/z 756.6. Following sequence interpretation and database searching, the MS/MS spectrum was matched to α_{s1} -casein f(109-114).

action of lactic acid bacteria. The identified peptides were chemically synthesized and their IC_{50} values were determined (Table 1). Five of the 16 identified peptides did not show ACE-inhibitory activity because their IC_{50} values were up to 1000 μM . Another 5 peptides showed moderate activity, with IC_{50} values varying from 342 to 465 μM . The C-terminal tripeptide residues of 3 of these peptides [β -CN f(94-105), β -CN f(203-209), and κ -CN f(119-123)] corresponded to zones of caprine proteins that differ from bovine and ovine sequences. As an example, peptide GPFILV, derived from caprine β -CN, contains Leu as the penultimate amino acid and its IC_{50} value was 424.0 μM . However, peptide LLYQQ-PVLGPVRGPFPIIV, released from bovine β -CN by hydrolysis with *Lactobacillus helveticus* CP790 proteinase, contains Ile at the penultimate position, and its IC_{50} value was 22 μM (Yamamoto et al., 1994). The differences between caprine and bovine sequences would be responsible for the different ACE-inhibitory activity shown by these peptides. Similarly, Kohmura

et al. (1990) had reported the important contribution of changes in structure of bovine, ovine, buffalo, rat, mouse, and human β -CN on ACE-inhibitory activity of peptides derived from them.

Six of the identified peptides showed potent ACE-inhibitory activity, with IC_{50} values ranging from 2.4 to 223.2 μM (Table 1). Two of them would be mainly responsible for the ACE-inhibitory activity of permeate from caprine kefir. The fragment f(58-68) of β -CN, LVYPFTGPIPN (IC_{50} value of 27.9 μM), showed high homology with a previously described peptide as a potent ACE inhibitor in a Gouda cheese (Saito et al., 2000). The most active peptide identified in our study, which had the sequence PYVRYL, corresponded to α_{s2} -CN f(203-208) and its IC_{50} value was 2.4 μM (Table 1). This activity was higher than that shown by peptides VPP and IPP, isolated from fermented milk with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (Nakamura et al., 1995). The ACE-inhibitory activity of peptide PYVRYL was even higher than that shown by

Table 1. Casein-derived peptides identified in the fractions collected by reverse phase-HPLC from the water-soluble extract of caprine kefir.

Fraction ¹	Observed mass	Calculated mass ²	Protein fragment	Sequence	IC ₅₀ , ³ μ M
F5.4	697.3	697.4 $\pm$ 0.06	α _{s1} -CN f(109-114)	LEIVPK	>1000
	755.8	755.4 $\pm$ 0.08	β -CN f(47-52)	DKIHHPF	>1000
	1038.3	1038.4 $\pm$ 0.02	β -CN f(48-56)	KIHHPFAQAQ	132.6 $\pm$ 14.1
F5.5	741.2	741.5 $\pm$ 0.08	α _{s1} -CN f(97-102)	QLLKLLK	342.4 $\pm$ 32.1
	958.2	958.3 $\pm$ 0.04	β -CN f(6-14)	LNVVGETVE	>1000
	1311.4	1311.5 $\pm$ 0.04	β -CN f(94-105)	GVPKVKETMVPK	376.1 $\pm$ 36.9
	1448.4	1448.5 $\pm$ 0.08	β -CN f(94-106)	GVPKVKETMVPKH	223.2 $\pm$ 21.3
F6	526.2	526.4 $\pm$ 0.04	κ -CN f(119-123)	IPAIN	432.6 $\pm$ 41.6
	741.4	741.5 $\pm$ 0.04	β -CN f(203-209)	GPFPIILV	424.0 $\pm$ 42.4
	775.3	775.5 $\pm$ 0.05	α _{s2} -CN f(174-179)	KFAWPQ	177.1 $\pm$ 14.9
	1022.3	1022.5 $\pm$ 0.02	β -CN f(63-72)	TGPIPNSLPQ	>1000
F7	425.1	425.2 $\pm$ 0.02	β -CN f(60-62)	YPF	>1000
	598.3	598.4 $\pm$ 0.04	β -CN f(50-54)	HPFAQ	465.0 $\pm$ 43.4
	790.3	790.4 $\pm$ 0.06	α _{s1} -CN f(18-23)	ENLLRF	82.4 $\pm$ 8.9
	809.3	809.4 $\pm$ 0.07	α _{s2} -CN f(203-208)	PYVRYL	2.4 $\pm$ 0.2
	1216.4	1216.5 $\pm$ 0.01	β -CN f(58-68)	LVYPFTGPIPN	27.9 $\pm$ 2.3

¹Fractions are termed as in Figure 1.²Monoisotopic mass values.³Protein concentration needed to inhibit 50% the original ACE activity. Results are presented as the mean (n = 3) $\pm$ SD.

the tetrapeptide VRYL (IC₅₀ value of 24.1 μ M), isolated from permeate of the water-soluble extract from 8-month-old Manchego cheese by Gómez-Ruiz et al. (2004). This fact could be due to the contribution of the dipeptide N-terminal ProTyr to the increased ACE-inhibitory activity of peptide PYVRYL.

Simulation of Gastrointestinal Digestion of Synthetic ACE-Inhibitory Peptides

To study the behavior of the peptides identified in caprine kefir under gastrointestinal digestion, the synthetic peptides were subjected to a 2-stage hydrolysis process, which simulated physiological conditions. The hydrolysates were analyzed by HPLC-MS/MS to identify the fragments released by the action of digestive enzymes (Table 2). The ACE-inhibitory activity of these hydrolysates was also tested and compared with that found for the nonhydrolyzed peptides (Table 2). Four of the 16 peptides were not hydrolyzed by digestive enzymes and thus remained intact in the final hydrolysates. The ACE-inhibitory activity of these hydrolysates did not change. Three of these peptides contained Pro in the penultimate position. This amino acid could increase the resistance of peptides to the action of proteolytic enzymes. Similarly, peptides identified in a Manchego cheese that contained Pro as a C-terminal residue or in a penultimate position survived the incubation with pepsin and the pancreatic extract (Gómez-Ruiz et al., 2004).

The peptide DKIHHPF was partially hydrolyzed by the digestive enzymes, and thus a small amount of this

peptide could be detected in the final hydrolysate. However, the major constituent of this hydrolysate was the fragment DKIHHP, released after action of the enzymes on the C-terminal bond of peptide DKIHHPF. The ACE-inhibitory activity increased notably after simulated digestion, and the IC₅₀ value of the final digest was 8 times lower than that found for the nonhydrolyzed peptide. The fragment DKIHHP that contains Pro as the C-terminal residue could be mainly responsible for the high activity observed. It is known that the Pro in this position enhances binding to the enzyme (Cheung et al., 1980). Another 3 peptides were also only partially hydrolyzed, but the ACE-inhibitory activity of the final digests was similar or slightly lower than that determined in the nonhydrolyzed peptides.

The other peptides identified in the active fractions from caprine kefir were fully hydrolyzed by digestive enzymes, releasing smaller fragments. Some of the peptides contained in the final digests were similar to or shared a great homology with peptides previously identified in our laboratory after simulated digestion of bovine fermented milks. As an example, the fragment GPFPI released from peptide GPFPIILV was also found in the hydrolysate of a commercial bovine fermented milk with pepsin and Corolase PP (Hernández-Ledesma et al., 2004a). Peptide VKETMVPK, contained in the hydrolysates of peptides GVPKVKETMVPK and GVPKVKETMVPKH, was similar, except for 2 amino acid residues, to the β -CN f(98-105) fragment that had previously been identified in bovine milk cultured with *Lactobacillus rhamnosus* and hydrolyzed with digestive

Table 2. Behavior of angiotensin-converting enzyme (ACE)-inhibitory activity of peptides obtained from 3-kDa permeate of caprine kefir-water soluble extract under simulation of gastrointestinal digestion. Sequence and ACE-inhibitory activity of peptides released after digestion.

Sequence	IC ₅₀ , mg/mL ¹		Degree of hydrolysis	Fragments released after digestion
	Before digestion	After digestion		
LEIVPK	>1	>1	Not hydrolyzed	LEIVPK
IPAIN	0.228 ± 0.022	0.228 ± 0.022	Not hydrolyzed	IPAIN
KFAWPQ	0.137 ± 0.011	0.137 ± 0.011	Not hydrolyzed	KFAWPQ
YPF	0.394 ± 0.038	0.394 ± 0.038	Not hydrolyzed	YPF
DKIHPP	>1	0.233 ± 0.023	Partly hydrolyzed	DKIHPP; DKIHPP
LNVVGETVE	>1	>1	Partly hydrolyzed	LNVVGETVE; LNVVGE; LNVV; NVVGE; NVVGETVE
GPFPILV	0.314 ± 0.031	0.677 ± 0.062	Partly hydrolyzed	GPFPILV; GPFPIL; GPFP
LVYPFTGPIPN	0.034 ± 0.003	0.068 ± 0.003	Partly hydrolyzed	LVYPFTGPIPN; LVYP; TGPIPN
KIHPPFAQAQ	0.138 ± 0.015	0.215 ± 0.013	Hydrolyzed	KIHP
QLLKLK	0.254 ± 0.024	>1	Hydrolyzed	KLK
GVPKVKETMVPK	0.493 ± 0.048	>1	Hydrolyzed	VKETMVPK; ETMVPK; TMVPK; VKET; PK
GVPKVKETMVPKH	0.323 ± 0.031	>1	Hydrolyzed	VKETMVPK; ETMVPK; TMVPK; VKET; PK
TGPIPNSLPQ	>1	>1	Hydrolyzed	SLPQ; TGPIPN
HPFAQ	0.278 ± 0.026	>1	Hydrolyzed	HPFA; FAQ
ENLLRF	0.065 ± 0.007	0.406 ± 0.044	Hydrolyzed	ENL
PYVRYL	0.002 ± 0.0001	0.086 ± 0.009	Hydrolyzed	PYV

¹Concentration of peptide needed to inhibit 50% the original ACE activity. Results are presented as the mean (n = 3) ± SD.

enzymes (Hernández-Ledesma et al., 2004b). A reduction in ACE-inhibitory activity was observed after digestion of these 8 peptides that were totally hydrolyzed. However, after hydrolysis of the most active sequence identified in caprine kefir, PYVRYL, the decrease of ACE-inhibitory activity was moderate (IC₅₀ value from 0.002 to 0.086 mg/mL). Only the tripeptide PYV was detected in the final digest. Loss of the C-terminal sequence RYL could be responsible for the decline in ACE-inhibitory activity. The importance of leucine as the C-terminal amino acid in ACE-inhibitory activity was confirmed by Gómez-Ruiz et al. (2004).

The ACE-inhibitory activity observed after digestion of synthetic peptides was similar to or lower than that of nonhydrolyzed peptides. However, several of the released peptides were di-, tri-, and tetrapeptides (Table 2). Probably, these small peptides would be more easily absorbed, reaching the peripheral target sites and producing their effect in vivo. The capacity of small peptides to pass the gastrointestinal tract has been described (Pihlanto-Leppälä, 2001).

In conclusion, this paper reports the ACE-inhibitory properties of kefir made from caprine milk. Sixteen peptides were identified and synthesized to evaluate their ACE-inhibitory activity. Two of these peptides, with the sequences PYVRYL and LVYPFTGPIPN, showed a potent ACE-inhibitory activity with IC₅₀ values of 2.4 and 27.9 μ M, respectively. In addition, the results of this work showed the influence of digestion on the formation of new ACE-inhibitory peptides. After simulated gastrointestinal digestion, ACE-inhibitory activity was similar to or slightly lower than that of nonhydrolyzed peptides. However, an important increase in

this activity was observed after simulated digestion of peptide DKIHPP [β -CN f(47-52)] that could be attributed to the most abundant fragment released, which had the sequence DKIHPP. Several of the peptides released after simulated digestion were smaller and could be good candidates for exhibiting an antihypertensive effect. Further studies using spontaneously hypertensive rats are in progress to prove the antihypertensive activity of the 2 most potent ACE-inhibitors identified in this study, that is, PYVRYL and LVYPFTGPIPN.

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REFERENCES

- Algaron, F., G. Miranda, D. Le Bars, and V. Monnet. 2004. Milk fermentation by *Lactococcus lactis* with modified proteolytic systems to accumulate potentially bio-active peptides. *Lait* 84:115–123.
- Alting, A. C., R. J. G. M. Meijer, and E. C. H. van Beresteijn. 1997. Incomplete elimination of the ABBOS epitope of bovine serum albumin under simulated gastrointestinal conditions of infants. *Diabetes Care* 20:875–880.
- Ashar, M. N., and R. Chand. 2004. Antihypertensive peptides purified from milks fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Milchwissenschaft* 59:14–17.

- Beshkova, D., E. D. Simova, Z. I. Simov, G. I. Frengova, and Z. N. Spasov. 2002. Pure cultures for making kefir. *Food Microbiol.* 19:537–544.
- Breci, L. A., D. L. Tabb, J. R. Yates, III, and V. H. Wysocki. 2003. Cleavage N-terminal to proline: Analysis of a database of peptide tandem mass spectra. *Anal. Chem.* 75:1963–1971.
- Cheung, H. S., F. L. Wang, M. A. Ondetti, E. F. Sabo, and D. W. Cushman. 1980. Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. *J. Biol. Chem.* 255:401–407.
- Corli Witthuhn, R., T. Schoeman, and T. Britz. 2004. Isolation and characterization of the microbial population of different South African kefir grains. *Int. J. Dairy Technol.* 57:33–37.
- Cushman, D. W., and H. S. Cheung. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* 20:1637–1648.
- Farnworth, E. R., and I. Mainville. 2003. Kefir: A fermented milk product. Pages 77–111 in *Handbook of Fermented Functional Foods*. E. R. Farnworth, ed. CRC Press, Boca Raton, FL.
- Gobbetti, M., P. Ferranti, E. Smacchi, F. Goffredi, and F. Addeo. 2000. Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4. *Appl. Environ. Microbiol.* 66:3898–3904.
- Gómez-Ruiz, J. A., M. Ramos, and I. Recio. 2002. Angiotensin-converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starters cultures. *Int. Dairy J.* 12:697–706.
- Gómez-Ruiz, J. A., M. Ramos, and I. Recio. 2004. Angiotensin-converting enzyme-inhibitory activity of peptides isolated from Manchego cheese. Stability under simulated gastrointestinal digestion. *Int. Dairy J.* 14:1075–1080.
- Hernández-Ledesma, B., L. Amigo, M. Ramos, and I. Recio. 2004a. Angiotensin converting enzyme inhibitory activity in commercial fermented products. Formation of peptides under simulated gastrointestinal digestion. *J. Agric. Food Chem.* 52:1504–1510.
- Hernández-Ledesma, B., L. Amigo, M. Ramos, and I. Recio. 2004b. Application of HPLC-MS/MS to the identification of biologically active peptides produced by milk fermentation and simulated gastrointestinal digestion. *J. Chromatogr. A.* 1049:107–114.
- Hertzler, S. R., and S. M. Clancy. 2003. Kefir improves lactose digestion and tolerance in adults with lactose maldigestion. *J. Am. Diet. Assoc.* 103:582–587.
- Kohmura, M., N. Kio, and Y. Ariyoshi. 1990. Inhibition of angiotensin converting enzyme by synthetic peptide fragments of various β -caseins. *Agric. Biol. Chem.* 54:1101–1102.
- Kuwabara, Y., S. Nagai, N. Yoshimitsu, I. Nakagawa, Y. Watanabe, and Y. Tamai. 1995. Antihypertensive effect of the milk fermented by culturing with various lactic acid bacteria and a yeast. *J. Ferment. Bioeng.* 80:294–295.
- Leclerc, P.-L., S. F. Gauthier, H. Bachelard, M. Santure, and D. Roy. 2002. Antihypertensive activity of casein-enriched milk fermented by *Lactobacillus helveticus*. *Int. Dairy J.* 12:995–1004.
- Marshall, V., W. M. Cole, and B. E. Brooker. 1984. Observations on the structure of kefir grains and the distribution of the microflora. *J. Appl. Bacteriol.* 57:491–497.
- Nakamura, Y., N. Yamamoto, K. Sakai, and T. Takano. 1995. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *J. Dairy Sci.* 78:1253–1257.
- Pihlanto-Leppälä, A. 2001. Bioactive peptides derived from bovine whey proteins: Opioid and ACE-inhibitory peptides. *Trends Food Sci. Technol.* 11:347–356.
- Saito, T., T. Nakamura, H. Kitazawa, Y. Kawai, and T. Itoh. 2000. Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda Cheese. *J. Dairy Sci.* 83:1434–1440.
- Simova, E., D. Beshkova, A. Angelov, T. S. Hristozova, G. Frengova, and Z. Spasov. 2002. Lactic acid bacteria and yeasts in kefir grains and kefir made from them. *J. Ind. Microbiol. Biotechnol.* 28:1–6.
- Sipola, M., P. Finckenberg, R. Korpela, H. Vapaatalo, and M. Nurminen. 2002. Effect of long-term intake of milk products on blood pressure in hypertensive rats. *J. Dairy Res.* 69:103–111.
- Takano, T. 1998. Milk-derived peptides and hypertension reduction. *Int. Dairy J.* 8:375–381.
- Vujicic, I. F., M. Vulic, and T. Könyves. 1992. Assimilation of cholesterol in milk by kefir cultures. *Biotechnol. Lett.* 14:847–850.
- Wszolek, M., A. Y. Tamime, D. D. Muir, and M. N. I. Barclay. 2001. Properties of kefir made in Scotland and Poland using bovine, caprine and ovine milk with different starter cultures. *Lebensm. Wiss. Technol.* 34:251–261.
- Yamamoto, N., A. Akino, and T. Takano. 1994. Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* 77:917–922.
- Yamamoto, N., M. Maeno, and T. Takano. 1999. Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *J. Dairy Sci.* 82:1388–1393.