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Angiotensin Converting Enzyme Inhibitory Activity in Commercial Fermented Products. Formation of Peptides under Simulated Gastrointestinal Digestion

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The angiotensin converting enzyme (ACE)-inhibitory activity of several commercial fermented milks was evaluated. Most of these products showed moderate inhibitory activity, but a few exceptions were detected. The high ACE-inhibitory activity found in some cases could be related to the origin of the milk. Two of these products were subjected to an enzymatic hydrolysis process, which simulates physiological digestion, to study the influence of digestion on ACE-inhibitory activity. The activity did not significantly change or increase during simulated gastrointestinal digestion. The peptides generated from one selected product during simulated digestion were sequenced by tandem spectrometry. Most peptides found at the end of the simulated digestion were released after 30 min of incubation with the pancreatic extract. This suggests that physiological digestion promotes the formation of active peptides from the proteins present in these fermented products. The potential ACE-inhibitory activity of the identified peptides is discussed with regard to their amino acid sequences.

KEYWORDS: ACE-inhibitory activity; peptides, fermented milk products; simulated gastrointestinal digestion; mass spectrometry

INTRODUCTION

It is accepted that food proteins may act as precursors of biologically active peptides with different physiological effects. Among these activities, the inhibition of the angiotensin converting enzyme (ACE) is one of the most comprehensively studied. ACE is a multifunctional enzyme associated with the rennin−angiotensin system, which regulates the peripheral blood pressure, where this enzyme catalyzes both production of the vasoconstrictor angiotensin II and inactivation of the vasodilator bradykinin. Thus, inhibition of ACE in the organism results in a lowering of blood pressure (*1*), although it may also influence different regulatory systems involved in inmmunodefense and nervous system activity (*2*).

ACE-inhibitory peptides can be released from the inactive precursor protein or oligopeptide in vivo, that is, during gastrointestinal digestion or in vitro during food processing. In addition to the use of isolated enzymes to prepare active hydrolysates, fermentation has already been proved as a successful strategy to produce ACE-inhibitory peptides. A representative example is the production of potent antihypertensive peptides (IPP and VPP) by milk fermentation with *Lactobacillus helveticus* and *Saccharomyces cerevisiae* (*3*). The antihypertensive effect of this fermented milk, which is commercialized in Japan (Calpis, Calpis Co. Ltd., Tokyo, Japan), has been demonstrated in spontaneously hypertensive rats and in a clinical study with hypertensive human subjects (*4*). Selected strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis* subsp. *cremoris* have also been used to produce fermented milks that contained ACE-inhibitory peptides (*5*). ACE-inhibitory peptides were also found in several ripened cheeses (*6*, *7*) (for a review, see ref *8*). These findings have led to the suggestion that fermented milk products containing ACE-inhibitory peptides may have a great potential as functional foods for hypertension prevention.

Most of the studies on the production of ACE-inhibitory peptides by fermentation are performed with selected strains of lactic acid bacteria because the type of starter is one of the main factors that influences the formation of these peptides in fermented milks. With the exception of Calpis sour milk, which is a traditional Japanese soft drink, there are few data available about the presence of ACE-inhibitory peptides in commercial fermented products (*9*, *10*). Moreover, although the formation of bioactive peptides derived from milk proteins by protein hydrolysis or fermentation has been extensively studied during the past decade (*8*), resistance of these peptides to gastrointestinal enzymes is poorly documented.

The aim of our study was to investigate the presence of ACE-inhibitory substances as naturally occurring components of commercial fermented milk products. In addition, some of these products were subjected to an enzymatic hydrolysis process, which simulates physiological digestion, to study the formation of peptides with this activity. The peptides generated from one selected product during simulated physiological digestion were

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sequenced by tandem spectrometry (MS/MS). The potential ACE-inhibitory activity of these peptides is discussed in relation to the structure of the peptides.

MATERIALS AND METHODS

Samples and Preparation of Sample Extracts. Fermented milks (FM-1 to FM-15) and fresh cheeses (FC-1 to FC-4) were purchased on the Spanish market. Water-soluble extract (WSE) of fermented products (solid or liquid) was obtained by centrifugation at 12000*g* for 20 min at 5 °C and by filtration through Whatman no. 40 filter. Most fermented milks were made from skim or half-skim milk with skim milk powder or other milk protein fractions and different lactic acid strains. LF-13 and the fresh cheeses were prepared by using rennet. Two liquid fermented milks (FM-5 and FM-8) were selected to simulate gastrointestinal digestion. FM-5 was prepared from half-skimmed milk, sugars, and different lactic acid bacteria including *Lactobacillus casei*, and FM-8 contained skim milk, powder skim milk, sugars, and different lactic acid bacteria.

Measurement of ACE-Inhibitory Activity. ACE-inhibitory activity was measured by the spectrophotometric assay of Cushman and Cheung (*11*) as modified by Kim et al. (*12*). Briefly, 20 μL of each sample was added to 0.1 mL of 0.1 M potasium phosphate buffer (pH 8.3) containing 0.3 M NaCl and 5 mM hippuryl-histidyl-leucine (Sigma Chemical Co., St. Louis, MO). ACE (5 milliunits) (EC 3.4.15.1, 5.1 units/mg, Sigma) was added, and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0.1 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 of products with ACE-inhibitory indices >50% was also calculated as the protein concentration needed to inhibit 50% of the original ACE activity (IC_{50}), and 1 unit of ACE-inhibitory activity was expressed as the potency showing 50% ACE inhibition under these conditions.

Total protein content of the WSEs was determined according to the Kjeldahl method. Aminic nitrogen was measured using the Cd−nynhidrin method according to Doi et al. (*13*). The peptidic nitrogen content was calculated as the total minus aminic nitrogen.

Analysis by On-line RP-HPLC-MS/MS. RP-HPLC separations of the WSEs were performed on an Agilent HPLC system connected on-line to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, Bremen, Germany). The HPLC system was equipped with a quaternary gradient pumping system, an in-line degasser, a variable-wavelength absorbance detector set at 220 nm, and an automatic injector (all 1100 series, Agilent Technologies, Waldbronn, Germany). The column used in these experiments was a 250 mm × 4.6 mm Widepore C_{18} column (Bio-Rad, Richmond, CA). The injection volume was 50 μL. Solvent A was a mixture of water/trifluoroacetic acid (1000:0.37 v/v), and solvent B contained acetonitrile/trifluoroacetic acid (1000:0.27 v/v). Peptides were eluted with a linear gradient of solvent B in A going from 0 to 45% in 60 min at a flow rate of 0.8 mL/min. The flow was split postdetector by placing a T-piece (Valco, Houston, TX) connected with a 75 μm i.d. peek outlet tube of an adjusted length to give ~20 μL/min of flow directed into the mass spectrometer via the electrospray interface. Nitrogen was used as nebulizing and drying gas and operated with an estimated helium pressure of 5×10^{-3} bar. The capillary was held at 4 kV. Spectra were recorded over the mass/charge (*m*/*z*) range of 100−2500. About 25 spectra were averaged in the MS analyses and about 5 spectra in the MS(*n*) analyses. The signal threshold to perform auto MS(*n*) analyses was 10000 (i.e., 5% of the total signal), and the precursor ions were isolated within a range of 4.0 *m*/*z* and fragmented with a voltage ramp going from 0.35 to 1.4 V. Using data analysis (version 3.0; Bruker Daltoniks), the *m*/*z* spectral data were processed and transformed to spectra representing mass values. BioTools (version 2.1; Bruker Daltoniks) was used to process the MS-(*n*) spectra and to perform peptide sequencing.

Simulation of Gastrointestinal Digestion. Two fermented milks (FM-5 and FM-8) were selected to simulate gastrointestinal digestion. These hydrolysates were prepared from an aqueous solution of product (0.7 wt % protein/vol). Hydrolysis was carried out according to the method of Alting et al. (*14*). The samples were first hydrolyzed with pepsin (EC 3.4.4.1; 1:60000, 3400 units/mg) (Sigma) (20 mg/g of protein) for 90 min at 37 °C at a pH of 2.0 followed by hydrolysis with Corolase PP (40 mg/g of protein) (Röhm, Darmstadt, Germany) at pH 7−8 and 37 °C for 240 min. Corolase PP is a proteolytic enzyme preparation from pig pancreas glands that besides trypsin and chymotrypsin contains numerous amino- and carboxylpeptidase activities. Hydrolysis was carried out in a thermally controlled water bath under constant stirring. Aliquots were withdrawn after hydrolysis with pepsin, the pH was raised to 7−8 with 1 M NaOH, and they were heated at 95 °C for 10 min in a water bath. During hydrolysis with Corolase PP, samples were also taken at 30, 120, and 240 min. The enzyme was inactivated by heating at 95 °C for 10 min, followed by cooling to room temperature. Each sample was stored at −20 °C until further analysis.

After the last samples had been taken, aliquots and the remaining reaction mixtures were centrifuged at 10000*g* for 30 min, and the supernatants were subjected to ultrafiltration through a hydrophilic 3000 Da cutoff membrane (Centripep, Amicon, Inc., Beverly, MA). The permeates were freeze-dried and kept at −20 °C until use.

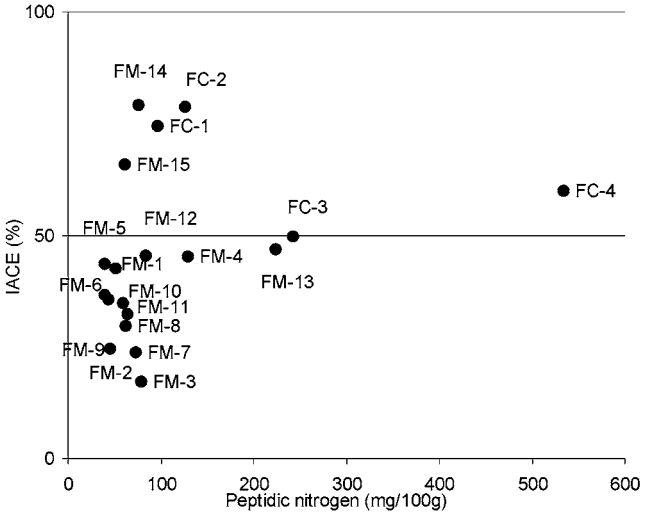

Figure 1. Values of the ACE-inhibitory indices versus peptide content expressed as milligrams of peptidic nitrogen per 100 g of product for the water-soluble extracts obtained from commercial fermented milks (FM) and fresh cheeses (FC).

RESULTS AND DISCUSSION

ACE-Inhibitory Activity of Commercial Fermented Products. In a first experiment, the ACE-inhibitory activity of the WSEs of several commercial fermented products was screened. Because the ACE-inhibitory activity of these products can be related to their peptide content, the peptidic nitrogen of these products was also calculated. **Figure 1** shows the ACE inhibition percentage versus peptidic nitrogen expressed as milligrams of nitrogen per 100 g of product. Most of the fermented products (12 from a total of 19) showed low ACE-inhibitory indices (<50%) and a peptide content that ranged between 39 and 150 mg of nitrogen/100 g of product. Only five products exhibited ACE-inhibitory indices >50%. Four of them contained levels of peptidic nitrogen similar to those of most of the fermented products analyzed in this study (i.e., values lower than 150 mg/100 g), but their ACE-inhibitory indices were significantly higher, ranging between 66 and 80%. The other product, which corresponded to a fresh cheese sample, showed a moderate ACE-inhibitory index (60%) and a relatively high proteolysis degree (533 mg of peptidic nitrogen/100 g). The ACE-inhibitory activity of the two fermented milks (FM-14 and FM-15) and

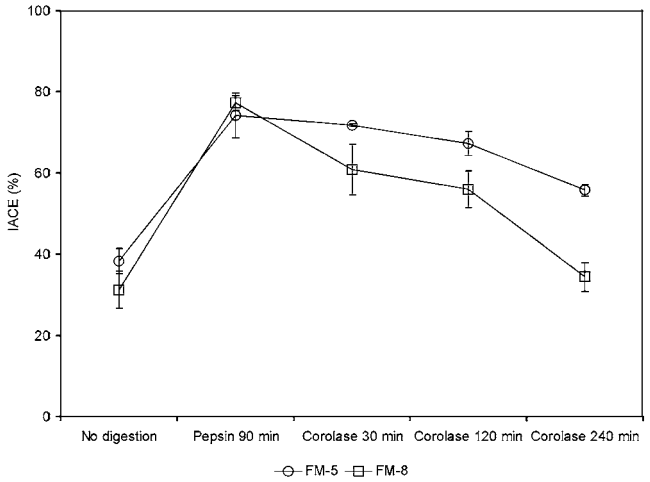

Figure 2. Evolution of the ACE-inhibitory indices of two fermented milks (FM-5 and FM-8) during simulated gastrointestinal digestion. Each point in the curve corresponds to an aliquot withdrawn during hydrolysis.

the two fresh cheeses (FC-1 and FC-2) with ACE-inhibitory indices >50% was also calculated as IC_{50}, that is, the concentration needed to inhibit 50% the original ACE activity. The IC_{50} values of FM-14, FM-15, FC-1, and FC-2 were 61.4 μg of peptidic nitrogen/mL (91 ACE-inhibitory units/mL), 16.9 μg of peptidic nitrogen/mL (270 ACE-inhibitory units/mL), 22.4 μg of peptidic nitrogen/mL (330 ACE-inhibitory units/mL), and 69.1 μg of peptidic nitrogen/mL (138 ACE-inhibitory units/mL), respectively. Interestingly, among these four fermented products, the fermented milks FM-14 and FM-15 corresponded to fermented products elaborated with milk of caprine origin, whereas the rest of the samples considered in this study were of bovine origin. Specifically, FM-14 corresponded to a Kefir-type product made from caprine milk cultured with several species of lactic and acetic acid bacteria and yeast. A Kefir-like fermented milk prepared with different lactic acid bacteria and yeast had previously been reported to exhibit an antihypertensive effect in spontaneously hypertensive rats, although it showed weak in vitro ACE inhibition (*15*). It has been postulated that fermented milk produced by mixing several types of microbes might contain a wider variety of functional substances than milk cultured with a single strain (*15*). In fact, Calpis sour milk, which is fermented with *Lactobacillus helveticus* and *Sacharomyces cerevisiae*, had been the source of two potent ACE-inhibitory peptides (*3*). Although the ACE-inhibitory indices found in this screening are moderate, they are higher than that previously reported for a commercial yogurt, for which the activity of the extract was <10% (*10*).

Hydrolysis under Simulated Gastrointestinal Conditions. To evaluate whether these fermented products may act as a natural source of ACE-inhibitory peptides produced by gastrointestinal enzymes, two products with low ACE-inhibitory indices (FM-5 and FM-8) were subjected to a two-stage hydrolysis process that simulates physiological digestion. During the pepsin-catalyzed part of the simulated physiological digestion, the ACE-inhibitory index of these two products increased sharply compared with the nonhydrolyzed products. The activity decreased gradually as digestion with Corolase PP progressed, but this reduction was less pronounced for FM-5 than for FM-8 (**Figure 2**). As a result, after simulated physiological digestion, the ACE-inhibitory activity of FM-5 was higher than that observed before digestion and higher than the activity found in the digested FM-8 sample. Similar activity values were found for FM-8 before and after hydrolysis (31 and 34%, respectively).

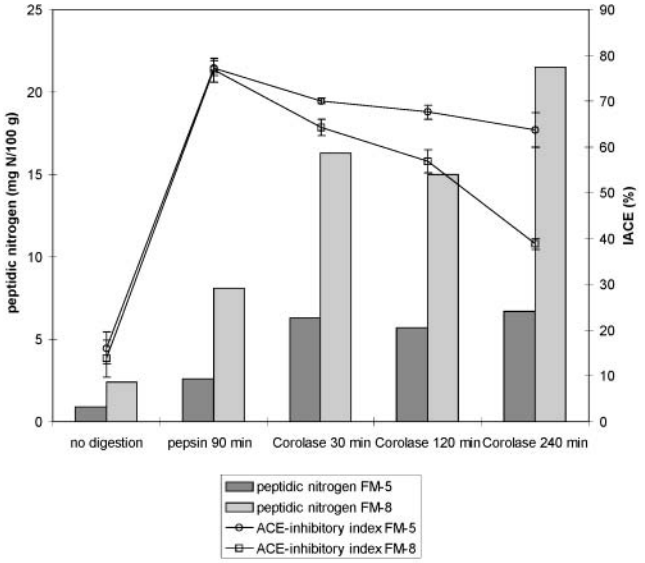

Figure 3. ACE-inhibitory indices (lines) and peptide contents (bars) expressed as milligrams of peptidic nitrogen per 100 g of product of the 3 kDa permeates from two fermented milks (FM-5 and FM-8) during simulated gastrointestinal digestion.

Because it has been suggested that small peptides make a considerable contribution to the ACE-inhibitory activity of protein hydrolysates (*16*) and short peptide sequences are good candidates to play a physiological antihypertensive role in vivo, ACE-inhibitory activity and the peptide content of permeates, obtained following ultrafiltration of the hydrolysates, were also assayed. The ACE-inhibitory activities of the 3 kDa permeates of FM-5 and FM-8 and their peptide contents throughout the simulated physiological digestion are shown in **Figure 3**. The inhibitory potency and the evolution of the ACE-inhibitory activity of the permeates during simulated digestion followed the same trend as that observed for the total hydrolysate. However, it has to be noted that the activity of the permeates at the end of digestion was higher than that observed prior to digestion for both fermented products. This can be explained by low ACE-inhibitory activity found in the permeates of the undigested products, which was approximately half the activity measured in the total undigested products. This result suggests that the ACE-inhibitory activity found in the undigested product was partially caused by peptides of masses >3 kDa. The peptide contents of FM-5 and FM-8 notably increased during the pepsin-catalyzed part of the digestion and after the first 30 min of hydrolysis with Corolase PP. Although the final ACE-inhibitory activity of the FM-5 permeate is higher than that found for FM-8, the peptide content of FM-5 over the digestion process was always lower than that of FM-8. This fact may indicate the presence of peptide sequences with higher ACE-inhibitory activity or higher concentration of active peptides in the hydrolysate of FM-5.

Identification of Peptides after Simulated Physiological Digestion. The 3 kDa permeate from FM-5 was subjected to RP-HPLC coupled on-line to a mass spectrometer in order to identify the peptide sequences obtained at the end of the simulated physiological digestion. In our case, the mass spectrometer was a quadrupole ion trap capable of multiple stages of mass analysis from a single precursor ion. As an example, **Figure 4** shows the MS/MS spectrum of a single charged ion with *m*/*z* 652.4 and the amino acid sequence of the identified peptide with the major fragment ions. Major fragment ions were observed at *m*/*z* 243.9 and 440.2 that corresponded

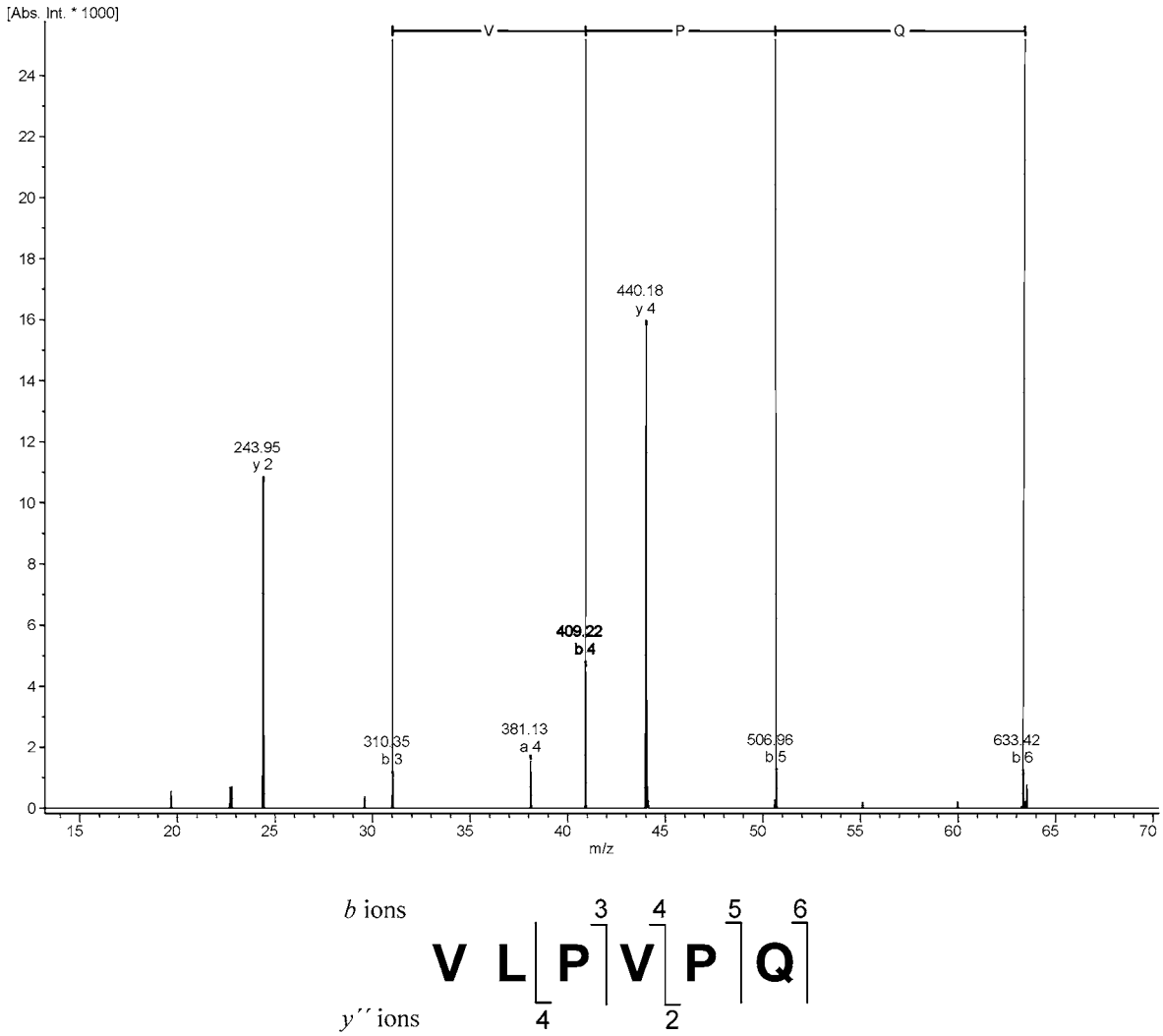

Figure 4. Tandem MS spectrum of ion *m*/*z* 652.4. Following sequence interpretation and database searching, the MS^2 spectrum was matched to β-casein f(170–175). The sequence of this peptide is displayed with the fragment ions observed in the spectrum. Fragment ions are labeled according to the nomenclature proposed by Roepstorff et al. (*29*).

to the *y*″-type ions adjacent to the amino acid proline. This amino acid is associated with very abundant *y*″-type ions, which are often easily identifiable because of their intensity (*17*). In some cases, some different charged ions from the same peptide were fragmented, allowing us to acquire additional information for peptide sequencing. All peptides of the total ion chromatogram with a signal >10000 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 milk protein hydrolysis and, thus, probably peptides from other origins are also present in this ultrafiltered hydrolysate.

A total of 56 peptide fragments were identified, of which 24 corresponded to β-casein fragments (**Table 1**), 19 to α_{s1}- and α_{s2}-casein fragments (**Table 2**), and 5 to κ-casein fragments; 7 were derived from the β-lactoglobulin sequence (**Table 3**). The dipeptide, RL (peptide 44, **Table 2**), could be originated from hydrolysis of various protein fractions. Of many ACE-inhibitory peptides, structure−activity correlation indicates that the C-terminal tripeptide residues play a predominant role in competitive binding to the active site of ACE. Among the most favorable C-terminal amino acids are aromatic amino acids, as well as the imino acid proline, whereas ACE only weakly binds peptides that have terminal dicarboxylic amino acids (*18*). It has to be stressed that several of the peptides identified in this study share the characteristic of having hydrophobic residues, such as leucine, phenylalanine, or valine, at C-terminal positions. Other peptides had lysine as the C-terminal residue (see, for instance, peptides 5−7 in **Table 1**). Structure relationship data suggest that many ACE-inhibitory peptides derived from milk proteins contain a positively charged amino acid as the C-terminal residue, which contributes substantially to the inhibitory potency (*19*).

Among the identified peptides, β-casein f(108−113) (peptide 7 in **Table 1**) had been previously identified by Pihlanto-Leppälä et al. as an ACE inhibitor in a casein sample fermented with lactic acid starters (*16*). This peptide showed moderate ACE-inhibitory activity with an IC_{50} value of 423 μg/mL. Moreover, some β-casein-derived peptides share some C-terminal residues with different ACE-inhibitory peptides previously described in the literature. For instance, β-caseins f(193−201) and f(196−201) (peptides 19 and 20 in **Table 1**) share the three terminal positions with the tripeptide GPV, which has been proved to have a potent in vitro ACE-inhibitory activity (IC_{50} = 1.2 μg/mL, 4.67 μM) (*20*). Although the activity of di- or tripeptides with ACE-inhibitory activity may not always be strictly extrapolated to longer peptides (*21*), the structural similarity of the C-terminal region may allow a similar activity to be

Table 1. β-Casein-Derived Peptides Identified in the 3 kDa Permeate Obtained from FM-5 after Simulated Physiological Digestion

	obsd mass	calcd mass[a]	protein fragment[b]	sequence	ACE-inhibitory peptides[c]	IC_{50} (ref)	peptide formation[d]
1	674.4	674.32	β-CN f(1–5)	RELEE			2, 3, 4, 5
2	512.3	512.28	β-CN f(49–52)	IHPF	DKIHPF	193.9 μg/mL (*5*)	4, 5
3	633.4	633.32	β-CN A^1 f(63–68)	PGPIHN	YPFPGPIPN (β–CN A^2)	14.8 μg/mL (*23*)	5
4	753.4	753.44	β-CN f(81–87)	PVVVPPF			3, 4, 5
5	872.5	872.48	β-CN f(98–105)	VKEAMAPK			3, 4, 5
6	645.4	645.32	β-CN f(100–105)	EAMAPK			3, 4, 5
7	747.4	747.36	β-CN f(108–113)	EMPFPK	EMPFPK	423 μg/mL (*16*)	3, 4, 5
8	603.4	603.29	β-CN f(114–118)	YPVEP			3, 4, 5
9	750.4	750.36	β-CN f(114–119)	YPVEPF			3, 4, 5
10	587.3	587.30	β-CN A^2 f(115–119)	PVEPF	MPFPKYPVQPF (β–CN A^1)	nr[e] (*23*)	5
11	1104.4	1103.57	β-CN f(124–133)	SLTLTDVENL			3, 4, 5
12	688.5	688.43	β-CN f(134–139)	HLPLPL	HLPLP	23.6 μg/mL (*24*)	3, 4, 5
13	551.4	551.37	β-CN f(135–139)	LPLPL	LPLP	315.6 μg/mL (*24*)	3, 4, 5
14	664.4	664.45	β-CN f(135–140)	LPLPLL			3, 4, 5
15	673.4	673.34	β-CN f(157–162)	FPPQSV			3, 4, 5
16	520.2	520.25	β-CN f(164–168)	SLSQS			2, 3, 4, 5
17	651.4	651.40	β-CN f(170–175)	VLPVPQ	SKVLPVPQ	39 μg/mL (*22*)	3, 4, 5
					PPQSVLSLSQSKVLPVPQ	25 μg/mL (*22*)	
18	503.3	503.24	β-CN f(179–182)	PYPQ			3, 4, 5
19	1000.4	1000.52	β-CN f(193–201)	YQEPVLGPV	GPV	1.2 μg/mL (*20*)	3, 4, 5
20	580.5	580.36	β-CN f(196–201)	PVLGPV	GPV	1.2 μg/mL (*20*)	3, 4, 5
21	685.5	685.39	β-CN f(202–207)	RGPFPI			3, 4, 5
22	897.5	897.54	β-CN f(202–209)	RGPFPIIV	LLYQQPVLGPVRGPFPIIV	21 μg/mL (*22*)	3, 4, 5
23	741.4	741.44	β-CN f(203–209)	GPFPIIV	LLYQQPVLGPV RGPFPIIV	21 μg/mL (*22*)	3, 4, 5
24	529.4	529.29	β-CN f(203–207)	GPFPI			3, 4, 5

[a] Monoisotopic mass. [b] β-Casein sequence according to ref *25*. [c] Previously described ACE-inhibitory peptides that share at least three C-terminal residues with those found in this study. [d] 1, undigested product; 2, digestion with pepsin for 90 min; 3, digestion with pepsin and Corolase PP for 30 min; 4, digestion with pepsin and Corolase PP for 120 min; 5, digestion with pepsin and Corolase PP for 240 min. [e] Not reported.

Table 2. α_{s1}- and α_{s2}-Casein-Derived Peptides Identified in the 3 kDa Permeate Obtained from FM-5 after Simulated Physiological Digestion

	obsd mass	calcd mass[a]	protein fragment[b]	sequence
25	399.3	399.26	α_{s1}-CN f(1–3)	RPK
26	493.4	493.30	α_{s1}-CN f(4–7)	HPIK
27	678.4	678.35	α_{s1}-CN f(8–13)	HQGLPQ
28	906.4	906.46	α_{s1}-CN f(8–15)	HQGLPQEV
29	904.5	904.47	α_{s1}-CN f(24–31)	FVAPFPEV
30	488.3	488.23	α_{s1}-CN f(27–30)	PFPE
31	507.4	507.28	α_{s1}-CN f(90–93)	RYLG
32	633.4	633.35	α_{s1}-CN f(104–108)	YKVPQ
33	484.4	485.25	α_{s1}-CN f(107–110)	PQLE
34	525.3	525.26	α_{s1}-CN f(125–129)	EGIHA
35	644.4	644.32	α_{s1}-CN f(133–138)	EPMIGV
36	520.2	520.25	α_{s1}-CN f(146–149)	YPEL
37	449.3	449.24	α_{s1}-CN f(150–152)	FRQ
38	374.3	374.18	α_{s1}-CN f(155–157)	QLD
39	910.4	910.46	α_{s1}-CN f(163–169)	AWYYVPL
40	788.5	788.44	α_{s2}-CN f(99–105)	LYQGPIV
41	1023.5	1023.62	α_{s2}-CN f(111–119)	QVKRNAVPI
42	398.3	399.21	α_{s2}-CN f(115–118)	NAVP
43	527.4	527.30	α_{s2}-CN f(118–122)	PITPT
44	287.1	287.20	various fragments	RL

[a] Monoisotopic mass. [b] α_{s1}- and α_{s2}-casein sequence according to refs *25* and *26*.

Table 3. κ-Casein- and β-Lactoglobulin-Derived Peptides Identified in the 3 kDa Permeate Obtained from FM-5 after Simulated Physiological Digestion

	obsd mass	calcd mass[a]	protein fragment[b]	sequence
45	495.3	495.23	κ-CN f(18–21)	FSDK
46	574.3	573.35	κ-CN f(20–24)	DKIAK
47	469.3	469.29	κ-CN f(26–29)	IPIQ
48	548.4	548.30	κ-CN f(61–65)	YAKPA
49	655.4	655.37	κ-CN f(106–111)	MAIPPK
50	443.9	444.30	β-Lg f(1–4)	LIVT
51	506.3	506.25	β-Lg f(5–8)	QTMK
52	273.1	273.18	β-Lg f(40–41)	RV
53	516.4	517.28	β-Lg f(44–47)	EELK
54	374.3	374.22	β-Lg f(83–85)	KID
55	576.2	575.24	β-Lg f(130–134)	DEALE
56	516.4	517.24	β-Lg f(155–158)	QLEE

[a] Monoisotopic mass. [b] κ-Casein and β-lactoglobulin sequence according to refs *27* and *28*.

anticipated. In the same manner, peptides β-casein f(202−209) and β-casein f(203−209) (peptides 22 and 23 in **Table 1**) are included in a previously described active sequence (IC_{50} = 21 μg/mL) from β-casein obtained by hydrolysis of casein with *Lactobacillus helveticus* CP790 proteinase. This casein hydrolysate had demonstrated not only in vitro ACE-inhibitory activity but also antihypertensive effect in spontaneously hypertensive rats after oral administration (*22*).

The MS/MS data permitted us to identify several peptides that belong to different genetic variants of β-casein. This is the case of β-casein f(63−68), for which our results agreed with the variant A^1 or peptide β-casein f(115−119), where we found the fragment corresponding to the A^2 variant. The amino acid changes due to the genetic variants of β-casein lead to a discrepancy between the two sequences found in this study and the same regions previously reported in the literature with ACE-inhibitory activity (peptides 3 and 10 in **Table 1**) (*23*).

Formation of the peptides of interest during simulated gastrointestinal digestion could easily be followed by analysis of the 3 kDa permeates by HPLC-MS and extraction of the characteristic ion of the peptide of interest. As an example, **Figure 5** shows the total ion current chromatogram corresponding to digested FM-5 and the extracted ion chromatograms of the molecular ion corresponding to β-casein f(170−175) (m/z 652.4). This β-casein fragment was also included in two previously described sequences, which exhibited potent ACE-inhibitory activity (**Table 1**) (*22*). This peptide was absent in the undigested product or after the pepsin-catalyzed part of the digestion (**Figure 5B**), but it was detected after 30 min of hydrolysis with Corolase PP (**Figure 5C**). The concentration

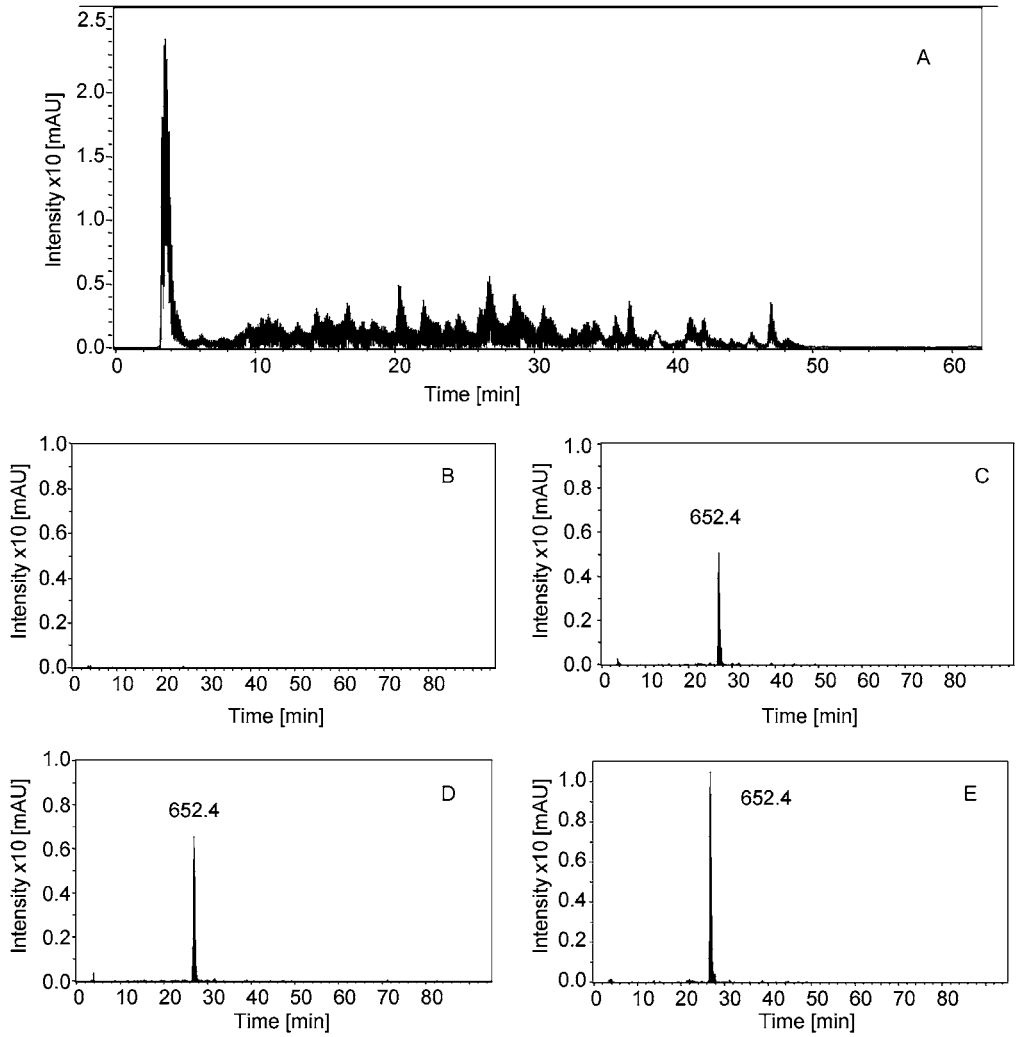

Figure 5. (**A**) Total ion current chromatogram corresponding to the 3 kDa permeate from sample FM-5 after simulated gastrointestinal digestion. Panels **B**–**E** correspond to the extracted ion chromatograms of ion *m*/*z* 652.4 after 30 min of digestion with pepsin (**B**) followed by 30 min of hydrolysis with Corolase PP (**C**), 120 min of hydrolysis with Corolase PP (**D**), and 240 min of hydrolysis with Corolase PP (**E**).

of this peptide increased with hydrolysis time to reach its maximum concentration after 240 min of hydrolysis with Corolase PP (**Figure 5E**). The same procedure was followed with other β-casein-derived sequences, and the results are summarized in **Table 1**. Except for the β-casein peptides f(49−52), f(63−68), and f(115−119) (peptides 2, 3, and 10 in **Table 1**) that appeared with longer incubation times with Corolase PP, most peptides found at the end of the simulated digestion were formed after 30 min of incubation with this enzyme. Only two peptides, β-casein f(1−5) and β-casein f(164−168) (peptides 1 and 16), which were detected after hydrolysis with pepsin, survived the treatment with the pancreatic extract.

In conclusion, this paper demonstrates the presence of moderate ACE-inhibitory activity in most of the fermented products considered. However, few commercial products showed potent activity, which could be related in some products to the origin of the milk. The ACE-inhibitory activity of these commercial products remained stable or increased after simulated gastrointestinal digestion. The successful strategy, by MS/MS sequencing and extraction of the ion of interest, allowed us to unambiguously identify peptides in the final digest of one selected product and to follow their formation during simulated physiological digestion. Most peptides found at the end of the simulated gastrointestinal digestion were formed during incubation with the pancreatic extract. Our results suggest that physiological digestion may promote the formation of active peptides from the proteins and oligopeptides present in commercial dairy products. Some of the identified peptides showed marked structural similarities with previously described ACE inhibitors.

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LITERATURE CITED

(1) Koike, H.; Ito, K.; Miyamoto, M.; Nishino, H. Effects of long-term blockade of angiotensin-converting enzyme with captopril (SQ 14,255) on hemodynamics and circulating blood volume in SHR. *Hypertension* **1980**, *2*, 229−303.

(2) Meisel, H. Casokinins as inhibitors of angiotensin-converting-enzyme. In *New Perspectives in Infant Nutrition*; Sawatzki, G., Renner, B., Eds.; Thieme Stuttgart: New York, 1993, pp 153−159.

(3) Nakamura, Y.; Yamamoto, N.; Saki, K.; Okubo, A.; Yamazaki, S.; Takano, T. Purification and characterisation of angiotensin I-converting enzyme inhibitors from sour milk. *J. Dairy Sci.* **1995**, *78*, 777−783.

(4) Takano, T. Milk derived peptides and hypertension reduction. *Int. Dairy J.* **1998**, *8*, 375−381.
(5) Gobbetti, M.; Ferranti, P.; Smacchi, E.; Goffredi, F.; Addeo, F. 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*. **2000**, *66*, 3898−3904.
(6) Ryhänen, E.-L.; Pihlanto-Leppälä, A.; Pahkala, E. A new type of ripened, low-fat cheese with bioactive properties. *Int. Dairy J.* **2001**, *11*, 441−447.
(7) Gómez-Ruiz, J. A.; Ramos, M.; Recio, I. Angiotensin-converting enzyme-inhibitory peptides in Manchego cheeses manufactured with different starters cultures. *Int. Dairy J.* **2002**, *12*, 697−706.
(8) Gobbetti, M.; Stepaniak, L.; De Angelis, M.; Corsetti, A.; Di Cagno, R. Latent bioactive peptides in milk proteins: Proteolytic activation and significance in dairy processing. *Crit. Rev. Food Sci. Nutr*. **2002**, *42*, 223−239.
(9) Okamoto, A.; Hanagata, H.; Matsumoto, E.; Kawamura, Y.; Koizumi, Y.; Yanagida, F. Angiotensin I converting enzyme inhibitory activities of various fermented foods. *Biotech. Biochem*. **1995**, *59*, 1147−1149.
(10) Meisel, H.; Goepfert, A.; Günther, S. ACE-inhibitory activities in milk products. *Milchwissenschaft* **1997**, *52*, 307−311.
(11) Cushman, D. W.; Cheung, H. S. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol*. **1971**, *20*, 1637−1648.
(12) Kim, Y. K.; Yoon, S.; Yu, D. Y.; Lönnerdal, B.; Chung, B. H. Novel angiotensin-I-converting enzyme inhibitory peptides derived from recombinant human a_{s1}-casein expressed in *Escherichia coli*. *J. Dairy Res.* **1999**, *66*, 431−439.
(13) Doi, E.; Shibata, D.; Matoba, T. Modified colorimetric ninhydrin methods for peptidase assay. *Anal. Biochem.* **1981**, *118*, 173−184.
(14) Alting, A. C.; Meijer, R. J. G. M.; van Beresteijn, E. C. H. Incomplete elimination of the ABBOS epitope of bovine serum albumin under simulated gastointestinal conditions of infants. *Diabetes Care* **1997**, *20*, 875−880.
(15) Kuwabara, Y.; Nagai, S.; Yoshimitsu, N.; Nakagawa, I.; Watanabe, Y.; Tamai, Y. Antihypertensive effect of the milk fermented by culturing with various lactic acid bacteria and a yeast. *J. Ferment. Bioeng*. **1995**, *80*, 294−295.
(16) Pihlanto-Leppälä, A.; Rokka, T.; Korhonen, H. Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *Int. Dairy J*. **1998**, *8*, 325−331.
(17) Papayannopoulus, A. I. The interpretation of collision-induced dissociation tandem mass spectra of peptide. *Mass Spectrom. Rev.* **1995**, *14*, 49−73.
(18) Cheung, H.-S.; Wang, F.-L.; Ondetti, M. A.; Sabo, E. F.; Cushman, D. W. Binding of peptide substrates and inhibitors of angiotensin-converting enzyme. Importance of the COOH-terminal dipeptide sequence. *J. Biol. Chem.* **1980**, *255*, 401−407.
(19) Meisel, H. Biochemical properties of regulatory peptides derived from milk proteins. *Biopolymers* **1997**, *43*, 119−128.
(20) Kim, S.-K.; Byun, H.-G.; Park, P. J.; Shahidi, F. Angiotensin I converting enzyme inhibitory peptides purified from bovine skin gelatin hydrolysate. *J. Agric. Food Chem*. **2001**, *49*, 2992−2997.
(21) Cushman, D. W.; Cheung, H. S.; Sabo, E. F.; Ondetti, M. A. Design of potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl and mercaptoalkanoyl amino acids. *Biochemistry* **1977**, *16*, 5484−5491.
(22) Yamamoto, N.; Akino, A.; Takano, T. Antihypertensive effect of the peptides derived from casein by an extracellular proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* **1994**, *77*, 917−922.
(23) Saito, T.; Nakamura, T.; Kitazawa, H.; Kawai, Y.; Itoh, T. Isolation and structural analysis of antihypertensive peptides that exist naturally in Gouda Cheese. *J. Dairy Sci*. **2000**, *83*, 1434−1440.
(24) Kohmura, M.; Nio, N.; Kubo, K.; Minoshima, Y.; Munekata, E. Inhibition of angiotensin-converting enzyme by synthetic peptides of human β-casein. *Agric. Biol. Chem.* **1989**, *53*, 2107−2114.
(25) Grosclaude, F.; Mahé, M.-F.; Mercier, J.-C.; Ribadeau-Dumas, B. Characterization of genetic variants of α_{s1}- and β-bovine caseins. *Eur. J. Biochem*. **1972**, *26*, 328−337.
(26) Brignon, G.; Ribadeau Dumas, B.; Mercier, J.-C.; Pelissier, J.-P.; Das, B. C. Complete amino acid sequence of bovine α_{s2}-casein. *FEBS Lett*. **1977**, *76*, 274−279.
(27) Mercier, J.-C.; Brignon, G.; Ribadeau-Dumas, B. Primary structure of bovine kappa B casein. Complete sequence. *Eur. J. Biochem*. **1973**, *35*, 222−235.
(28) Braunitzer, G.; Chen, R.; Schrank, B.; Stangl, A. The sequence of β-lactoglobulin. *Hoppe-Seyler's Z. Physiol. Chem*. **1973**, *354*, 867−878.
(29) Roepstorff, P.; Fohlman, J. Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom*. **1984**, *11*, 601.

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