JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2004,** *52,* 6923−6931 **6923**

Identification of Angiotensin-I-Converting Enzyme Inhibitory Peptides Derived from Sodium Caseinate Hydrolysates Produced by *Lactobacillus helveticus* NCC 2765

MARIE-CLAUDE ROBERT,*,† ALAIN RAZANAME,§ MANFRED MUTTER,§ AND MARCEL A. JUILLERAT†

Nestlé Research Center, Nestec Ltd., P.O. Box 44, 1000 Lausanne 26, Switzerland, and Institute of Chemical Sciences and Engineering, Ecole Polytechnique Fédérale de Lausanne, 1015 Lausanne, Switzerland

Angiotensin-I-converting enzyme (ACE) inhibitory activity was identified in milk proteins fermented with *Lactobacillus* (*Lb.*) *helveticus* NCC 2765 (Nestlé Culture Collection, Vers-chez-les-Blanc, Switzerland). Hydrolyzing sodium caseinate for 1 and 2 h inhibited ACE activity, as measured by an in vitro ACE inhibition test. The hydrolysates with the highest ACE inhibitory potential were fractionated by gel permeation chromatography and their low molecular weight fractions collected. These fractions were subsequently subfractionated by reverse-phase high-pressure liquid chromatography. Several hydrophobic subfractions showed high ACE inhibitory potential, and their peptide composition was determined using an ion trap mass spectrometer equipped with an elctrospray ionization source. Analysis of the low molecular weight fraction identified 14 peptides with known antihypertensive activity and 1 with previously described opioid activity. On the basis of the peptide composition of active subfractions, two potentially active novel sequences were defined, and the following synthetic peptides were synthesized: FVAPFPEVFG (α_{S1} 39−48), ENLLRFFVAPFPEVFG (α_{S1} 33−48), NENLLRFFVAPFPEVFG (α_{S1} 32−48), LNENLLRFFVAPFPEVFG (α_{S1} 31−48), NLHLPLPLL (β 147−155), ENLHLPLPLL (β 146−155), and VENLHLPLPLL (β 145−155). The ACE inhibitory potential of these synthetic peptides was assessed, and IC_{50} values were determined. NLHLPLPLL (β 147−155), which was the only synthetic peptide also present in the sodium caseinate hydrolysates, and NENLLRFFVAPFPEVFG (α_{S1} 32−48) showed the highest inhibition of ACE activity, with IC_{50} values of 15 and 55 μM, respectively. Furthermore, the stability of all synthetic peptides was assessed using an in vitro model simulating gastric digestion. The β-casein-derived peptides remained intact following the successive hydrolysis by pepsin and pancreatin, whereas α_{S1}-casein-derived peptides were degraded by pepsin.

KEYWORDS: ACE inhibitory peptides; milk; fermented caseins

INTRODUCTION

The multifunctional angiotensin-I-converting enzyme (ACE, peptidyldipeptide hydrolase, peptidyl dipeptidase A, EC 3.4.15.1) is located in several organs (e.g., plasma, lung, kidney, liver, testes, heart, muscle, brain, uterus, and intestine) (*1*) and influences different homeostatic systems of the organism. However, ACE is classically associated with the renin−angiotensin system and characterized as a key enzyme in the regulation of peripheral blood pressure. The broad substrate specificity of this glycoprotein explains its successive denomination as ACE, because it cleaves the carboxyl-terminal dipeptide from angiotensin I to produce the potent vasopressor octapeptide angiotensin II, and as a kininase II, because it inactivates bradykinin by the sequential removal of two carboxyl-terminal dipeptides (*2*).

Several endogenous peptides such as enkephalins, bradykinin, and substance P have been described as competitive substrates for angiotensin I (*3*), but exogenous inhibitors are more numerous. Exogenous ACE inhibitors displaying an antihypertensive effect in vivo were first discovered in snake venom (*4*); however, many proteins such as those in fish (*5*), gelatin (*6*), maize (*7*), plasma (*8*), rapeseed (*9*), soy sauce (*10*), soybean (*11*), soy milk (*12*), chicken muscle (*13*), chicken egg yolks (*14*), wheat germ (*15*), red alga (*16*), wakame (*17*), sake (*18*), yam tuber (*19*), royal jelly (*20*), and buckwheat (*21*) have been also been demonstrated to contain ACE inhibitory peptides.

Milk proteins, a principle source of bioactive peptides, are also precursors for numerous ACE inhibitors such as those

* Corresponding author (telephone +41 21 7858040; fax +41 21 7858553; e-mail marie-claude.robert@rdls.nestle.com).
† Nestec Ltd.
§ Ecole Polytechnique Fédérale de Lausanne.

10.1021/jf049510t CCC: $27.50 © 2004 American Chemical Society
Published on Web 10/16/2004

peptides from cheese (*22*), whey proteins (*23*), or caseins (*24*) and mainly from fermented milks (*25*, *26*). Such peptides may be released from milk proteins through the proteolytic activity of lactic acid bacteria. Lactobacilli, and in particular *Lactobacillus* (*Lb.*) *helveticus*, have been shown to have especially high proteolytic activity in comparison to other lactic acid bacteria (*27*, *28*). Two studies have shown that the extracellular cell wall protease of lactic acid bacteria was able to hydrolyze caseins into hundreds of oligopeptides (*29*, *30*). Chabance et al. (*31*) showed that a casein-derived peptide of 24 amino acids could cross the intestinal barrier and was identified in the plasma of human subjects 20 min after milk ingestion. Roberts et al. (*32*) additionally claimed that small (di- and tripeptides) and large (10−51 amino acids) peptides generated in the diet could be absorbed intact through the intestine and show biological effects in tissues. In vivo studies have demonstrated that feeding fermented milk rich in ACE inhibitory peptides could lower systolic blood pressure in spontaneously hypertensive rats (*3*, *33*). In humans suffering from mildly elevated blood pressure, daily ingestion of a Japanese fermented milk (Calpis skim milk fermented with *Lb. helveticus* and *Saccharomyces cervisiae*) over a period of 8 weeks led to a significant decrease in blood pressure (*34*).

In the present study, we used a specific *Lb. helveticus* strain to produce fermented milk containing ACE inhibitory peptides. Novel ACE inhibitory peptides were isolated, sequenced, and chemically synthesized. Their biological activity was characterized, and their resistance to hydrolysis by gastrointestinal peptidases was assessed.

MATERIALS AND METHODS

Chemicals. Sodium caseinate was supplied by DMW International (De Melkindustrie Veghel, Veghel, The Netherlands) as raw material. Unless stated otherwise, all chemicals used were of analytical grade and purchased from either Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). MRS broth was obtained from Difco Laboratories (Detroit, MI). Sterile filters GPWP and Ultrafree-0.5 centrifugal filter units were from Millipore (Bedford, MA). ACE kit, ACE calibrator pepsin (2500−3500 units per mg of protein), and pancreatin (activity equivalent to 4× U.S.P) were from Sigma-Aldrich (Buchs, Switzerland). Fmoc-amino acids were purchased from Bachem AG (Bubendorf, Switzerland).

Culture of Lactic Acid Bacteria. *Lb. helveticus* NCC 2765 (Nestlé Culture Collection, Vers-chez-les-Blanc, Switzerland) were grown overnight without shaking in sterile MRS broth at 40 °C. The culture was inoculated at 2% in ultrahigh-temperature (UHT) skim milk (purchased from retail outlets). Fermentation at 37 °C was stopped as the pH of the hydrolysate dropped below 4.5, and it was then readjusted to 5.2 with 1 N NaOH. Two percent trisodium citrate was added to the hydrolysates and the solution allowed to equilibrate for 10 min. Bacteria were centrifuged at 3700*g* and 4 °C for 10 min, washed three times with 30 mM Tris−maleate buffer, pH 7.5, and resuspended in the same buffer.

Caseinate Hydrolysis. Bacteria (8×10^9 bacteria per mL, final concentration) were incubated at 37 °C with 4 mg L^{-1} sodium caseinate. Samples were taken after 1, 2, and 5 h. The bacteria were removed by centrifugation at 14000*g*, and the proteolysis was stopped. The supernatant was recovered, and the samples were stored at −20 °C before further analysis.

Gel Permeation Chromatography (GPC) Separation of Amino Acids, Peptides, and Proteins. An Agilent (Palo Alto, CA) 1050 series module and system for HPLC was used for GPC. Instrument control, data processing, and analysis were performed using Agilent Chemstation Base software. All solvents were sterile filtered and purged by sonication. GPC was performed using a Superdex 75 HR 10/30 column (Amersham Pharmacia Biotech, Duebendorf, Switzerland). Isocratic elution was performed under non-denaturating conditions at a flow rate of 0.5 mL min^{-1} for 65 min using 50 mM ammonium acetate, pH 7. The detection was performed at 215 and 280 nm. Fractions were collected as a function of their molecular weight, dried, and resuspended in H_2O twice before final lyophilization. Because 100 μL of caseinate hydrolysate had been fractionated on the Superdex 75 column, the resulting lyophilized GPC fractions were resuspended in 100 μL of H_2O for further analysis.

Reverse-Phase High-Pressure Liquid Chromatography (RP-HPLC) Using C8 Column. Peptides separated by GPC methods were further fractionated using a SpectraSystem HPLC (Thermo Finnigan, San Jose, CA). The HPLC system consisted of a quaternary pump (TSP P4000), an autosampler (TSP AS3000), and a UV−vis detector (UV−vis 205 Linear) equipped with a high-pressure flow cell (1.6 μL volume, 2 mm path length). Instrument control was performed using XCalibur software (Thermo Finnigan). All solvents were filtered using sterile filters and purged by sonication. RP-HPLC was performed using a C8 column (208TP54, Vydac, Hesperia, CA). Solvent A (0.05% TFA in H_2O, v/v) and solvent B (0.045% TFA, 80% aqueous acetonitrile, v/v) formed the eluent in the following linear gradient steps: after 5 min of isocratic elution at 100% A, from 0 to 50% B over 60 min, then to 100% B over 1 min; after 4 min of isocratic elution at 100% B, to 100% A over 1 min; and finishing with 9 min of equilibration at 100% A before the next run was started. Total run time was 80 min, and a flow rate of 0.8 mL min^{-1} was applied. Peak detection was achieved at 215 nm. The injection volume was 100 μL. Following chromatographic separation, fractions were collected every 2.5 min (2 mL volume). The collected fractions were lyophilized and resuspended in H_2O twice before final lyophilization.

RP-HPLC Using C18 Column. Synthetic peptides that were hydrolyzed with pepsin and pancreatin were analyzed using a SpectraSystem HPLC (Thermo Finnigan), described above. All solvents were sterile and purged by sonication as indicated above. RP-HPLC was performed using a C18 column (Nucleosil C18, Macherey-Nagel AG, Oensingen, Switzerland). Solvent A (0.05% TFA in H_2O, v/v) and solvent B (0.045% TFA, 80% aqueous acetonitrile, v/v) formed the eluent in the following linear gradient steps: from 0 to 70% B over 15 min, then to 100% B over 30 s, after 90 s of isocratic elution at 100% B to 100% A over 30 s; and finishing with 11.5 min of equilibration at 100% A before the next run was started. Total run time was 30 min, and a flow rate of 0.8 mL min^{-1} was applied. Peak detection was at 215 nm. The injection volume was 50 μL.

Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS). Peptides were characterized using a Spectra System HPLC with a reverse-phase column (either C8 or C18) coupled to the Finnigan LCQ ion trap mass spectrometer equipped with an ESI source. If peptides had to be collected, HPLC flow was directed via 0.005 in. i.d. PEEK tubing to a micro flow-splitter with 10% going to the MS and 90% being collected in a fraction collector. The MS instrument was tuned and calibrated using the manufacturer's protocols (Thermo Electron, Waltham, MA). The ESI source was operated at 4.5 kV, and the interface capillary heater was set to 200 °C. Sheath gas flow was maintained at flow rates of 50 mL min^{-1}. All spectra were obtained in positive mode and recorded at unit-mass resolution. Automated MS/MS spectra were acquired with relative collision energy for collision-induced dissociation (CID) preset at 35% and an isolation width of 1 *m*/*z* units. Dynamic exclusion allowed the generation of MS/MS spectra of peptide mixtures in peaks not fully resolved by chromatography. The scan range was set at *m*/*z* 150.0−2000.0.

Data Analysis. Full scan MS, MS/MS data acquisition, and analysis were performed with Xcalibur software V1.0 SR1 (Thermo Electron), including the Bioworks V1.0 software package for SEQUEST database searches. The peptide masses that were obtained were compared to the SwissProt protein database using PeptIdent (Peptide Mass Fingerprinting) and PeptideMass programs accessible through the ExPASy Molecular Biology Server (http://www.expasy.ch). MS/MS spectra analysis was performed using the SEQUEST program. It correlates uninterpreted MS/MS spectra of peptides with amino acid sequences from protein and nucleotide databases. On the basis of this correlation the software determines the amino acid sequence and thus associates it with the protein(s) that correspond to the mass spectrum being analyzed.

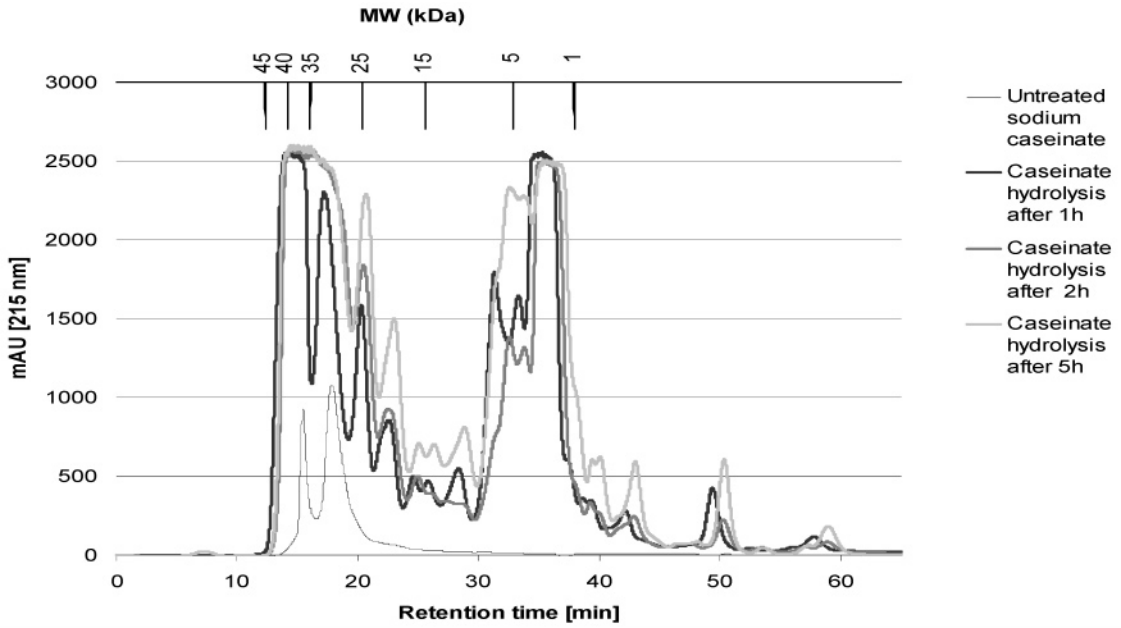

Figure 1. Gel permeation chromatograms of caseinate samples (corresponding to 400 μg of initial intact sodium caseinate) hydrolyzed for either 1, 2, or 5 h with *Lb. helveticus* NCC 2765. Reference material: 200 μg of untreated sodium caseinate.

Peptide Synthesis Procedure. All peptides (400−500 mg) were synthesized on an Advanced Chemtech peptide synthesizer ACE 348 omega using Fmoc-amino acids. Prior to peptide synthesis, the resin was swollen in dichloromethane (DCM) during 30 min. Peptides were assembled by sequential amino acid coupling on Sasrin resin applying the Fmoc strategy (*35*). According to the published procedures of automated peptide synthesis, a deprotection/coupling cycle comprises a 10 min deprotection and the coupling of the Fmoc-amino acid derivative (2 equiv) in DMF with benzotriazol-1-yloxytris(pyrrolidino)-phosphonium hexafluorophosphate (PyBOP, 2.5 equiv) as coupling reagent and *N*,*N*-diisopropylethylamine (DIEA, 5 equiv) as base. Cleavage of the peptide from the resin was performed with 2% trifluoroacetic acid (TFA) in DCM during 20 min. After treatment, the resin was separated by filtration and the filtrate concentrated in vacuo. For complete removal of the side-chain protection, the peptide was treated with a cocktail of TFA/triisopropylsilan (TIS)/water (90:5:5, v/v). After 1 h, cold diethyl ether was added to precipitate the product. The precipitate was collected by centrifugation and lyophilized from a water/acetonitrile (1:1, v/v) solution to afford a colorless solid. After lyophilization, the crude product was purified by preparative RP-HPLC on a C18 column by applying a linear gradient from 20 to 60% A (A, 0.9% TFA in acetonitrile; B, 10% A in H_2O) over 30 min at flow rate of 80 mL min^{-1}. The eluate was monitored by UV absorption at a wavelength of 214 nm, and appropriate fractions were collected and lyophilized. The peptide was characterized by electrospray ionization mass spectrometry (ESI-MS) and analytical RP-HPLC.

Angiotensin-Converting Enzyme Inhibitory Test. An ACE kit and ACE calibrator were used to assess inhibition. The procedure used here is a rapid spectrophotometric method based on the hydrolysis of the synthetic tripeptide substrate *N*-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycylglycine (FAPGG) to furylacryloylphenylanlanine (FAP) and glycylglycine, resulting in a decrease in absorbance at 340 nm. The ACE reagent and the ACE calibrator were reconstituted according to the manufacturer's recommendations. The assay was performed in quartz cuvettes by mixing 0.45 mL of ACE reagent solution, 0.05 mL of casein hydrolysate, and 0.05 mL of ACE calibrator and incubating at 37 °C for 10 min. The absorbance at 340 nm was measured as a function of time. All assays were performed in triplicate, and ACE activity was determined by comparing the sample reaction rate to that obtained with the ACE calibrator (control). The results were expressed as a percentage of the residual activity compared to the ACE calibrator alone.

In Vitro Method Estimating Protein Digestion. This procedure was used to mimic in vivo conditions of the gastrointestinal tract. One milligram of protein or peptide was dissolved in 400 μL of H_2O. HCl (2.5 μL, 1 N) was added until the pH of the solution reached pH 2.0. This solution was hydrolyzed for 1 h in a Thermomixer at 37 °C and 250 rpm with 1.5 μg of pepsin. The pH of the solution was neutralized with NaOH (3 μL, 1 N). The volume was then adjusted to 500 μL with H_2O, and a further digestion was carried out for 2 h in a Thermomixer at 37 °C and 250 rpm with pancreatin. Enzymes were finally removed from the hydrolysate using Ultrafree-0.5 centrifugal filter units having a nominal molecular mass limit of 10000 Da. Samples were centrifuged at 14000*g* for 30 min, according to manufacturer's recommendations.

RESULTS

Fermentation of caseins with *Lb. helveticus* NCC 2765 was reproducible ($n = 3$), as demonstrated by similar SDS-PAGE gel electrophoretic patterns (data not shown) and ACE-inhibiting activities equivalent to those described under Effect of Fermentation Time on ACE Inhibition of Hydrolysates. Low molecular weight peptide characterization of a hydrolysate was performed only once.

Fractionation of the Caseinate Hydrolysates. Fractionation of caseinate hydrolysates was performed in two steps: first, by GPC under non-denaturing conditions, and, second, by RP-HPLC. **Figure 1** depicts the size exclusion chromatograms of the caseinate hydrolysates following 1, 2, and 5 h of hydrolysis with *Lb. helveticus* NCC 2765. An equivalent quantity of each hydrolysate was injected, corresponding to 400 μg of initial intact sodium caseinate, whereas a 200 μg sample was injected for untreated sodium caseinate. Untreated sodium caseinate had a retention time of 17.8 min. As the GPC was performed under non-denaturing conditions, the additional peak observed at 15.4 min was attributed to peptide aggregates. Medium and low molecular weight peptides were accumulated with increasing hydrolysis time. Potential ACE-inhibiting small peptides in the range of 1−5 kDa were generated in largest amounts after 5 h of hydrolysis.

RP-HPLC was used in combination with mass spectrometry for the characterization of the sample. The mass spectrometric analysis and collection of fractions were performed in the same run, allowing the determination of the exact qualitative peptidic

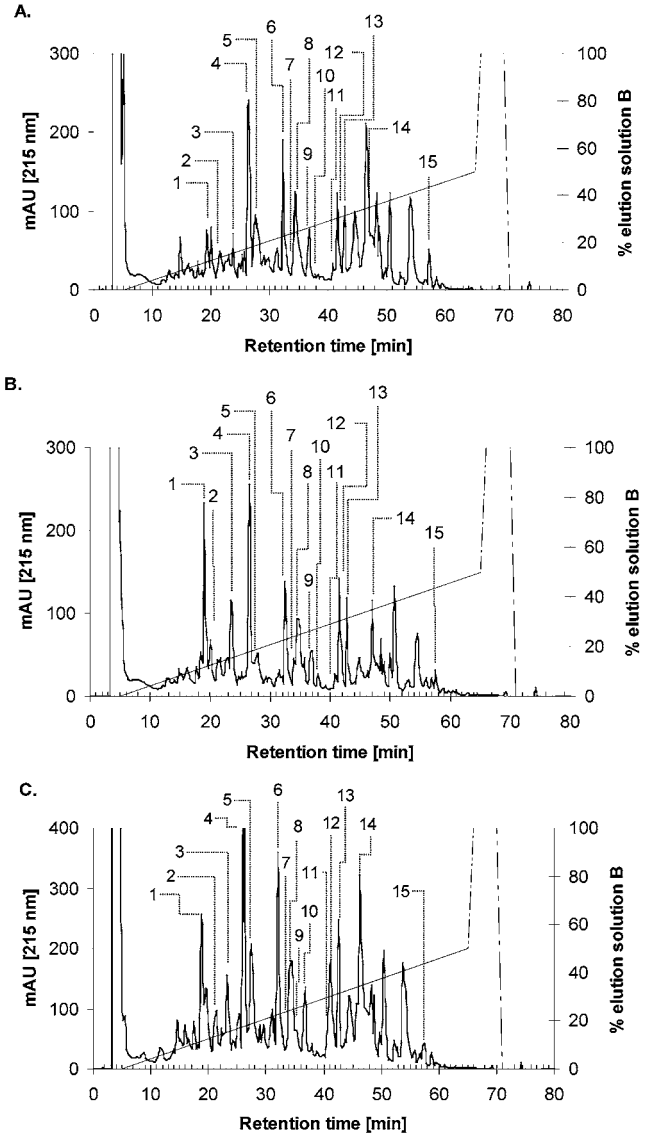

Figure 2. C8-RP-HPLC chromatograms of fractions containing peptides hydrolyzed for either 1 (**A**), 2 (**B**), or 5 (**C**) h with a molecular mass between 1 and 5 kDa. Peak numbers refer to peptides listed in **Table 1**.

composition of each collected fraction. As seen in **Figure 2**, the amount of low molecular weight peptides increased and changed as a function of time. The peaks in the chromatograms of peptides in the range of 1−5 kDa were distributed evenly over the whole chromatogram, showing an equal proportion of hydrophilic and hydrophobic peptides.

Effect of Fermentation Time on ACE Inhibition of Hydrolysates. As shown in **Figure 3**, caseinate hydrolyzed by *Lb. helveticus* for 1 and 2 h strongly inhibited ACE activity. We observed that the ACE-inhibiting activity of the 5 h hydrolysate was less than that of the unhydrolyzed caseinate. This suggests that ACE-inhibiting factors were generated during caseinate hydrolysis and that these factors, when hydrolyzed for 5 h, lost their ability to inhibit ACE activity.

Identification of Casein Peptides with Known Bioactivity. **Figure 2** represents the analytical RP-HPLC chromatogram of casein peptides hydrolyzed for either 1 (**A**), 2 (**B**), or 5 (**C**) h with a mass of 1−5 kDa. The peak numbers in **Figure 2** are described in **Table 1**. Fourteen peptides with known ACE inhibitory activity and 1 with opioid activity (β-casein 75−83) were identified with ESI-MS/MS (**Table 1**). Peptides were

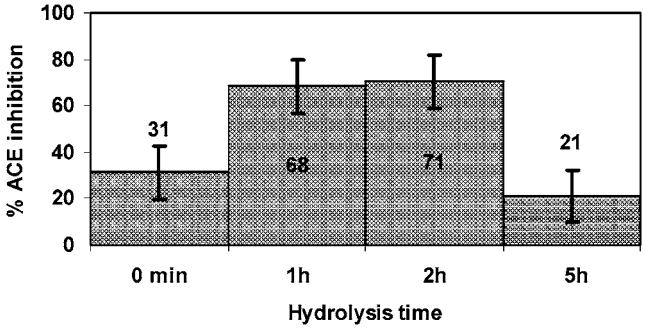

Figure 3. Effect of caseinate hydrolysates on the inhibition of ACE. Results are expressed as percentage of OD (OD measured at 340 nm) with respect to the control. All entries are averages of $n = 3$ independent determinations.

characterized by matching the uninterpreted MS/MS spectra obtained by CID of the protonated molecular ion with the predicted spectra for casein peptides. Fragmentation patterns with no clear assignable b- or y-type ions were not interpreted and rejected.

ACE Inhibition by Caseinate Hydrolysate Fractions. Following GPC, peptide fractions with a molecular mass of <5 kDa were subfractionated and further analyzed using LC-MS. These new subfractions were then tested for their ACE inhibitory activity. Few subfractions derived from caseinate hydrolyzed for 1 h (**Figure 4**) and 2 h (data not shown) were able to significantly inhibit in vitro ACE activity. As shown in **Figure 4**, RP-HPLC subfractions with retention times of 60−67.5 min were particularly active, demonstrating an ACE inhibition of nearly 75% compared to the control. These subfractions were analyzed by LC-MS, and 10 peptides were identified in the 1 h hydrolysate (**Table 2**). Five β-casein-derived peptides (LHLPLPLL, NLHLPLPLL, VENLHLPLPL, DVENLHLPLPLLQSW, and KYPVEPFTESQSLTLTDVENLHL) and two α_{S1}-casein-derived peptides (FVAPFPEVFGKEKVNELSKDIGS and NENLLRFFVAPFPE) showed a common amino acid sequence. The 2 h hydrolysate yielded subfractions [retention time (RT) = 60−67.5 min] that also significantly inhibited the ACE activity. From the 2 h hydrolysate, four peptides showing the β-casein sequence (LHLPLPLL, NLHLPLPLL, NLHLPLPLLQSW, and DVENLHLPLPLLQSW) as well as five peptides showing the α_{S1}-casein sequence (ENLLRFFVAPFPE, NENLLRFFVAPFPE, LNENLLRFFVAPVPEV, NENLLRFFVAPFPEVF, and ENLLRFFAPFPEVFGKEKV) were characterized. We defined two potentially ACE inhibitory peptide sequences: NLHLPLPLL on β-casein (147−155) and FVAPFPE on α_{S1}-casein (39−45).

Peptide Synthesis. To identify the minimal length of peptide required to inhibit ACE, several analogues were synthesized. From α_{S1}-caseins, we decided to synthesize the motif FVAPFPE (α_{S1} 39−45), the shortest fragment with a putative ACE inhibitory activity, and N-terminally elongated versions thereof: the motifs ENLLRFFVAPFPE (α_{S1} 33−45, one amino acid missing at the N terminus in comparison with the peptide found in the ACE-inhibiting subfractions), NENLLRFFVAPFPE (α_{S1} 32−45), the peptide found in the ACE-inhibiting subfractions), and a longer version LNENLLRFFVAPFPE (α_{S1} 31−45). For chemical synthesis purposes, the α_{S1}-casein sequence was elongated with three amino acids (V-F-G) at the C terminus. From β-casein, the peptides NLHLPLPLL (β 147−155), an N-terminal-elongated version ENLHLPLPLL (β 146−155), and a longer version VENLHLPLPLL (β 145−155) were synthesized.

Table 1. ESI-MS Results of Peptides Found in Caseinate Hydrolyzed by *Lb. helveticus* NCC 2765 and Described as Biologically Active in the Literature

RP-HPLC peak[a]	retention time (min)	peptide sequence	origin	ACE IC_{50} value (μM) (ref)	measured value (Da)	calcd value (Da)
2	21.1	LQSW	β-casein 155–158	500 (*52*)	533.2	532.6
11	39.9	HLPLP	β-casein 149–153	41 (*51*)	576.3	575.7
5	27.7	AVPYPQ	β-casein 192–197	>1000 (*48*)	674.3	673.8
3	23.8	RPKHPI	α_{S1}-casein 16–21	>1000 (*48*)	747.8	746.9
10	37.2	YQEPVL	β-casein 208–213	280 (*50*)	748.3	747.9
8	34.3	DKIHPF	β-casein 62–67	257 (*53*)	756.4	755.9
6	32.5	KVLPVPQ	β-casein 184–190	>1000 (*53*)	780.4	780.0
4	26.5	KAVPYPQ	β-casein 191–197	>1000 (*48*)	802.6	801.9
7	33.5	SKVLPVPQ	β-casein 183–190	39 (*48*)	867.4	867.1
12	41.7	RDMPIQAF	β-casein 198–205	209 (*48*)	977.5	977.2
13	42.5	YPFPGPIPN	β-casein 75–83		1001.4	1001.3
9	36.7	MKPWIQPK	α_{S2}-casein 205–212	300 (*52*)	1027.5	1027.3
1	19.3	RPKHPIKHQ	α_{S1}-casein 16–24	>1000 (*48*)	1140.7	1140.4
14	47.0	HKEMPFPKYPVEPF	β-casein 121–134	>1000 (*48*)	1746.1	1746.1
15	57.5	YQEPVLGPVRGPFPIIV	β-casein 208–224	101 (*48*)	1881.7	1881.3

[a] Peak numbers are from **Figure 2**.

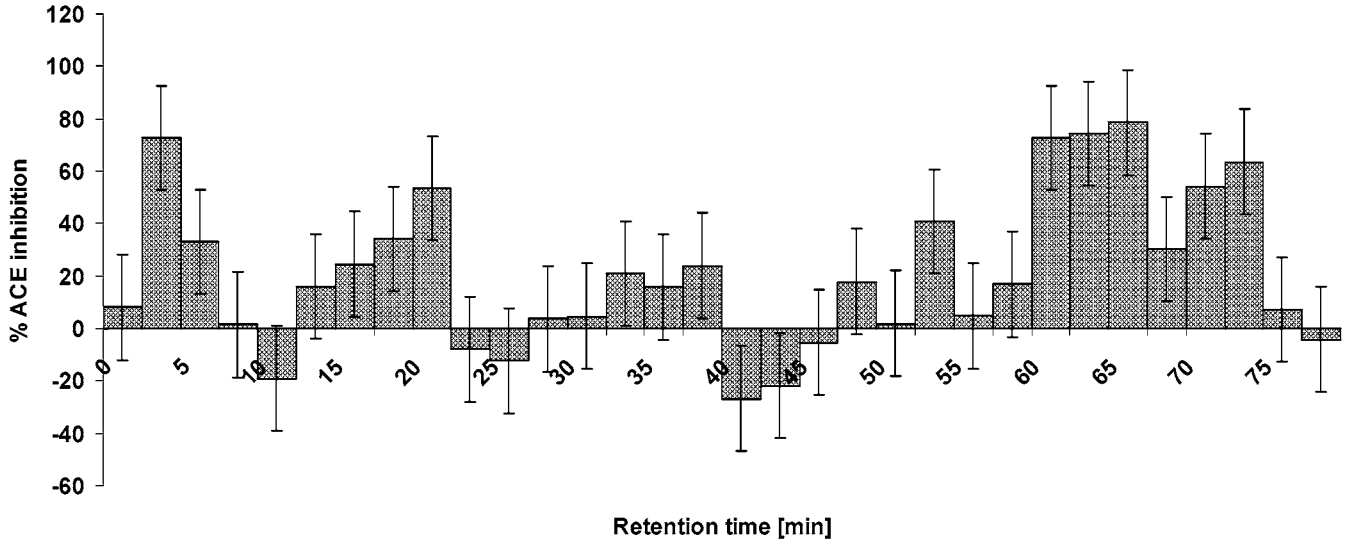

Figure 4. Effect of C8-RP-HPLC subfractions of the low molecular weight fraction of caseinate hydrolyzed for 1 h on the inhibition of ACE. Results are expressed as a percentage of OD (OD measured at 340 nm) with respect to the control. All entries are means of independent determinations ($n = 3$).

Table 2. Peptides Identified in Hydrophobic C8-RP-HPLC Subfractions, Retention Time from 59 to 67.5 min

retention time (min)	peptide sequence	origin	measured value (Da)	calcd value (Da)
59.3	LHLPLPLL	β-casein 148–155	916.2	915.2
59.6	NLHLPLPLL	β-casein 147–155	1030.3	1029.3
59.8	VENLHLPLPL	β-casein 145–154	1145.4	1144.4
62.5	DVENLHLPLPLLQSW	β -casein 144–158	1775.1	1774.1
62.5	KYPVEPFTESQSLTLTDVENLHL	β-casein 128–149	2662.0	2261.0
60.3	FVAPFPEVFGKEKVNELSKDIGS	α_{S1}-casein 39–61	2538.9	2538.0
60.3	NENLLRFFVAPFPE	α_{S1}-casein 32–45	1692.8	1693.0
60.0	LGPVRGPFPI	β-casein 213–222	1053.3	1052.3
59.7	QKAVPYPQRDMPI	β-casein 190–202	1543.8	1542.8
60.5	LPQYLKTVYQHQKA	α_{S2}-casein 191–204	1718.0	1717.0

In Vitro Inhibition of ACE. As shown in **Table 3**, the best IC_{50} values were measured for NLHLPLPLL (β 147−155) (IC_{50} = 15 μM) and for NENLLRFFVAPFPEVFG (α_{S1} 32−48) (IC_{50} = 55 μM). These results are in the range of IC_{50} values found for most food-derived antihypertensive peptides (*36*), which were obtained using Hip-His-Leu as substrate. Binevski et al. (*37*) found comparable K_m values for somatic and testicular bovine ACE using FAPGG and Hip-His-Leu as substrates. We assumed that the K_m values of rabbit lung ACE would also be similar for both substrates.

Active Peptides Resist Simulated Digestion. To have an antihypertensive function in the human body, the active peptides must resist gastric digestion and are absorbed from the intestinal lumen. It is known that small peptides such as di- and tripeptides

Table 3. IC_{50} for the Inhibition of ACE Using the Synthetic Tripeptide Substrate *N*-[3-(2-Furyl)acryloyl]-L-phenylalanylglycylglycine

		IC_{50} value (μM)
derived from β-casein	NLHLPLPLL	15
	ENLHLPLPLL	250
	VENLHLPLPLL	175
derived from α_{S1}-casein	FVAPFPEVFG	650
	ENLLRFFVAPFPEVFG	250
	NENLLRFFVAPFPEVFG	55
	LNENLLRFFVAPFPEVFG	280

are passively absorbed in the intestine; however, evidence exists that this may also hold true for larger peptides (*32*). To assess the possibility that bioactive peptides derived from casein can survive their passage through the digestive tract without being hydrolyzed, synthetic peptides VENLHLPLPLL (β 145−155), ENLHLPLPLL (β 146−155), NLHLPLPLL (β 147−155), LNENLLRFFVAPFPEVFG (α_{S1} 31−48), NENLLRFFVAPFPEVFG (α_{S1} 32−48), and ENLLRFFVAPFPEVFG (α_{S1} 33−48) were sequentially hydrolyzed with pepsin (1 h) and with pancreatin (2 h). Resulting hydrolysates were analyzed on a C18 column by RP-HPLC in conjunction with ESI-MS detection. Only peptides derived from β-caseins (VENLHLPLPLL, ENLHLPLPLL, and NLHLPLPLL) remained intact following their exposure to pepsin and pancreatin. In **Figure 5**, the ion chromatogram of ENLHLPLPLL after hydrolysis (**Figure 6A**) and its corresponding MS/MS fragmentation data (**Figure 6B**) are shown. The fragmentation pattern permitted the identification of the ENLHLPLPLL peptide, which was able to resist digestion by gastric enzymes. Pepsin hydrolyzed α_{S1}-casein-derived peptides (LNENLLRFFVAPFPEVFG, NENLLRFFVAPFPEVFG, and ENLLRFFVAPFPEVFG) between the two phenylalanine residues. The ion chromatogram of NENLLRFFVAPFPEVFG (α_{S1} 32−48) after hydrolysis and the corresponding MS/MS fragmentation data of NENLLRF and the MS spectrum of FVAPFPEVFG are shown in **Figure 6**. The ion at *m*/*z* 1109.5 was identified as the single-charged $[M + H]^+$ ion of the FVAPFPEVFG peptide. As this ion was charged only once, the MS/MS spectrum obtained was of limited quality (data not shown). The second fragmentation pattern showed b- and y-type ions that were characteristic for NENLLRF, confirming the hydrolysis between the two phenylalanine residues (NENLLRF•FVAPFPEVFG). These peptides were not tested for ACE inhibition.

DISCUSSION

Many studies have been performed on bioactive peptides that are present in milk proteins (*38*−*42*). These bioactive peptides are inactive within the sequence of the parent protein and are released and activated by enzymatic proteolysis, for example, by the action of proteinases and peptidases from lactic acid bacteria (*39*). In milk, caseins are the major source for amino acids, and it was demonstrated that the extracellular cell wall-associated proteinase of a lactic acid bacteria was able to hydrolyze this compound into more than 100 different oligopeptides (*29*). Consequently, lactic acid bacteria generate a large number of peptides, including those with potentially bioactive properties.

The data reported here demonstrate the ACE-inhibiting potential of sodium caseinate hydrolysates. Caseinate solutions were hydrolyzed for different times with *Lb. helveticus* NCC 2765 and the peptides characterized. Some hydrophobic subfractions showed high ACE-inhibiting activity, and their corresponding peptides were determined by LC-MS. On the basis of the peptide composition of these subfractions, two novel active sequences were identified and peptides of various lengths synthesized. The ACE inhibitory potential of these synthetic peptides was assessed, and IC_{50} values were determined. Results were comparable to the IC_{50} values of food-derived antihypertensive peptides (*36*). Finally, the stability of these peptides was assessed by successive hydrolysis with pepsin and pancreatin. Only β-casein-derived peptides remained intact after the sequential digestion with pepsin and pancreatin, whereas α_{S1}-casein-derived peptides were hydrolyzed by pepsin and partially lost their ACE inhibitory activity. In agreement with many authors (*25*, *26*, *33*, *34*, *43*−*49*), we demonstrated that a strong proteolytic starter strain was able to generate caseinate hydrolysates with significant ACE inhibitory activity. Pihlanto et al. (*50*), using yogurt, "ropy milk", and soured milk starter strains, could detect antihypertensive agents in fermented milk only after hydrolysis with trypsin and pepsin. Consequently, the proteolytic activity of the starter strain and the specificity of its extracellular proteinase are of importance, as the peptide pattern and

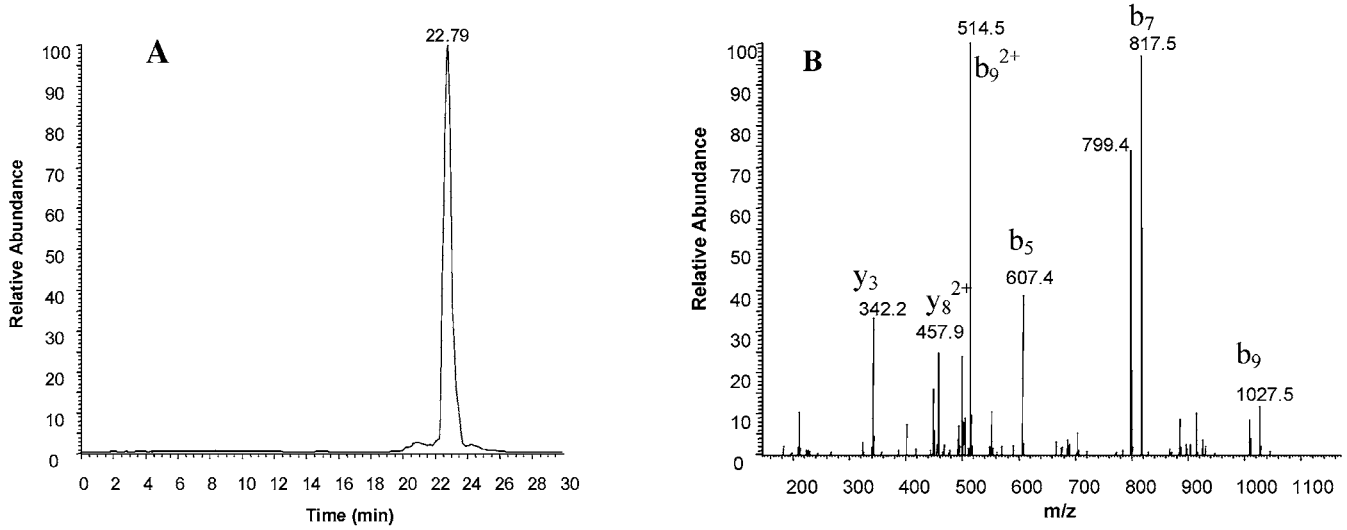

Figure 5. (**A**) Total ion current profile of the C18-RP-HPLC/ESI-MS of synthetic peptide ENLHLPLPLL (β 146–155) after in vitro gastric digestion. (**B**) MS/MS fragment ion mass spectrum of *m*/*z* 579.9, the double-charged $[M + H]^{2+}$ ion.

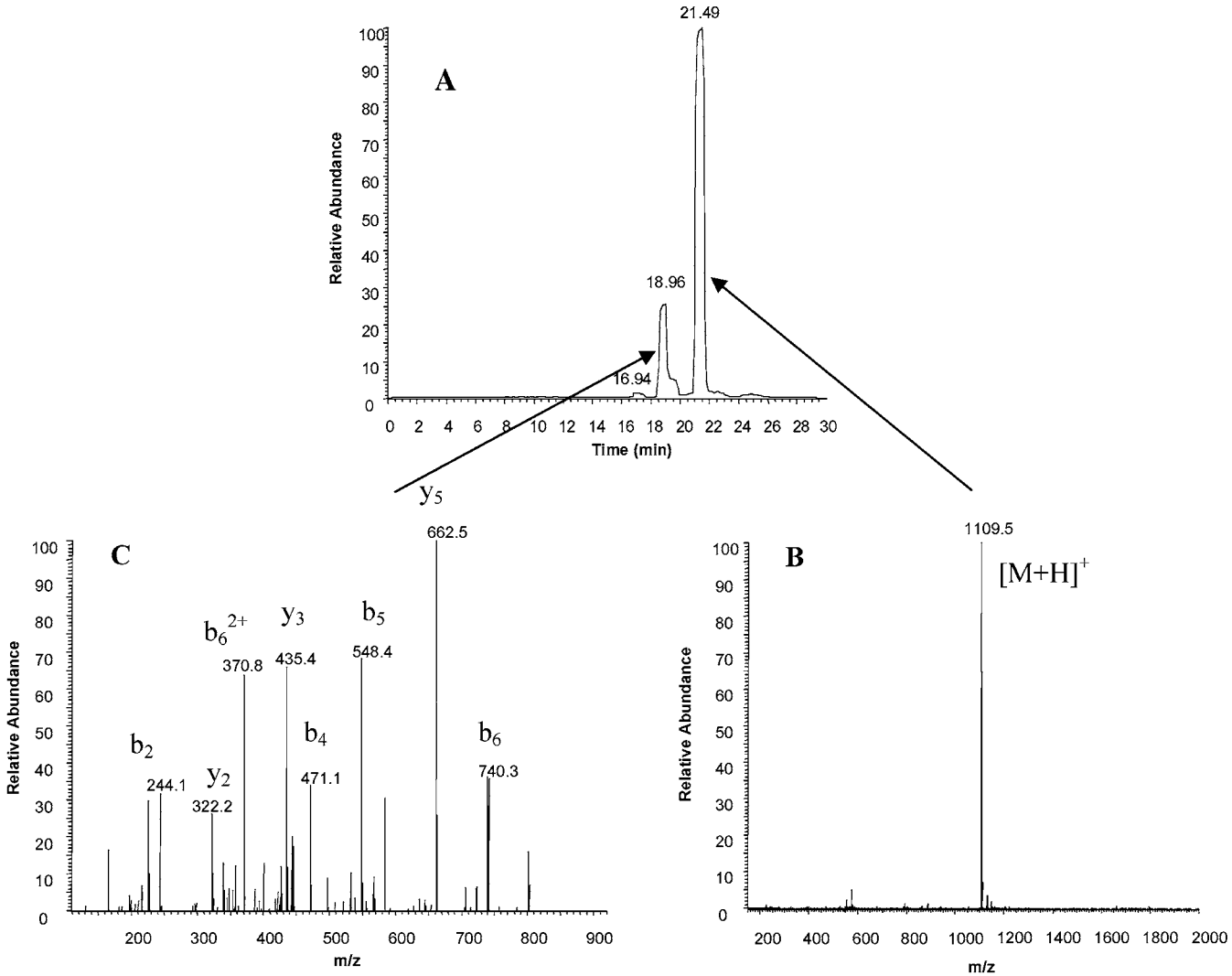

Figure 6. (**A**) Total ion current profile of the C18-RP-HPLC/ESI-MS of synthetic peptide NENLLRFFVAPFPEVFG (α_{S1} 32–48) after in vitro gastric digestion. (**B**) MS/MS fragment ion mass spectrum of *m*/*z* 1109.5, the single-charged $[M + H]^+$ ion derived from FVAPFPEVFG. (**C**) MS/MS fragment ion mass spectrum of *m*/*z* 453.3, the double-charged $[M + H]^{2+}$ ion derived from NENLLRF.

especially the kinetics of hydrolysis are strain-specific. Preferential cleavage sites of the protease from *Lb. helveticus* used in this study were determined by analyzing the C terminus of low molecular weight peptides generated throughout the hydrolysis of sodium caseinate. Our data agreed with the preferential cleavage sites described by Zevaco and Gripon (*30*), who showed hydrophobic (leucine), aromatic (phenylalanine), basic (lysine), or hydrophilic (glutamine) residues are the preferred targets at the N-terminal side of the scissile bond.

Inhibition of ACE activity is highly dependent on the structure. Structure−activity relationships of various peptide inhibitors indicated that the C-terminal tripeptide residues have an important role by competitively binding to the active site of ACE. Adding glutamic acid to the sequence NLHLPLPLL (bovine β 147−155) increased the IC_{50} value from 15 to 250 μM. The presence of an acidic residue at the N terminus seems to increase the IC_{50} value. A similar observation was made by Kohmura et al. (*51*), who measured an IC_{50} value of 51 μM for NLHLPLP (human β 137−143), which increased to 155 μM for ENLHLPLP (human β 136−143). Adding a single asparagine to ENLLRFFVAPFPEVFG (α_{S1} 33−48) decreased the IC_{50} value from 250 to 55 μM.

In conclusion, we have evaluated the ACE inhibitory activity of both fermented casein-derived peptides and synthetic variants in vitro. From these studies, we conclude that β-casein peptides have the greatest potential to modulate antihypertensive activity.

ABBREVIATIONS USED

ACE, angiotensin converting enzyme; CID, collision-induced dissociation; Da, daltons; DCM, dichloromethane; DIEA, *N*,*N*′-diisopropylethylamine; DMF, *N*,*N*′-dimethylformamide; ESI-MS, electrospray ionization mass spectrometry; FAP, furylacryloylphenylanlanine; FAPGG, *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine; Fmoc, fluorenyl methoxy carbonyl; GPC, gel permeation chromatography; Hip-His-Leu, *N*-benzoylglycyl-L-hystidyl-L-leucine; HPLC, high-pressure liquid chromatography; IC_{50}, concentration that causes 50% enzyme inhibition; kDa, kilodaltons; *Lb.*, *Lactobacillus*; MS, mass spectrometry; Mw, molecular weight; PyBOP, benzotriazol-1-yloxytris(pyrrolidino)phosphonium hexafluorophosphate; RP-HPLC, reverse-phase high-pressure liquid chromatography; rpm, revolutions per minute; TFA, trifluoroacetic acid; TIS, triisoproylsilan; Tris, tris(hydroxymethyl)aminomethane; U.S.P, U.S. Pharmacopeia; UV−vis, ultraviolet−visible; v/v, volume per volume.

ACKNOWLEDGMENT

We are grateful to Lucienne Juillerat-Jeanneret-Gris and David M. Mutch, who contributed to the manuscript with useful corrections and criticisms.

LITERATURE CITED

(1) Danilov, S. M.; Faerman, A. I.; Printseva, O. Y.; Martynov, A. V.; Sakharov, I. Y.; Trakht, I. N. Immunohistochemical study of angiotensin-converting enzyme in human tissues using monoclonal antibodies. *Histochemistry* **1987**, *87*, 487−490.

(2) Ryan, J. W. Angiotensin I converting enzyme (Kininase II). In *Enzymes 3: Peptidases, Proteinases and Their Inhibitors*; Bergmeyer, H. U., Ed.; Verlag Chemie: Weinheim, Germany, 1984.

(3) Maruyama, S.; Mitachi, H.; Tanaka, H.; Tomizuka, N.; Susuki, H. Studies on the active site and antihypertensive activity of angiotensin I-converting enzyme inhibitors derived from casein. *Agric. Biol. Chem.* **1987**, *51*, 1581−1586.

(4) Ferreira, S. H.; Bartelt, D. C.; Greene, L. J. Isolation of bradykinin-potentiating peptides from *Bothrops jararaca* venom. *Biochemistry* **1970**, *9*, 2583−2593.

(5) Curtis, J. M.; Dennis, D.; Waddell, D. S.; MacGillivray, T.; Ewart, H. S. 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*, 3919−3925.

(6) 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.

(7) Yano, S.; Suzuki, K.; Funatsu, G. Isolation from alpha-zein of thermolysin peptides with angiotensin I-converting enzyme inhibitory activity. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 661−663.

(8) Wanasundara, P. K.; Ross, A. R.; Amarowicz, R.; Ambrose, S. J.; Pegg, R. B.; Shand, P. J. Peptides with angiotensin I-converting enzyme (ACE) inhibitory activity from defibrinated, hydrolyzed bovine plasma. *J. Agric. Food Chem.* **2002**, *50*, 6981−6988.

(9) Marczak, E. D.; Usui, H.; Fujita, H.; Yang, Y.; Yokoo, M.; Lipkowski, A. W.; Yoshikawa, M. New antihypertensive peptides isolated from rapeseed. *Peptides* **2003**, *24*, 791−798.

(10) Kinoshita, E.; Yamakoshi, J.; Kikuchi, M. Purification and identification of an angiotensin I-converting enzyme inhibitor from soy sauce. *Biosci., Biotechnol., Biochem.* **1993**, *57*, 1107−1110.

(11) Kuba, M.; Tanaka, K.; Tawata, S.; Takeda, Y.; Yasuda, M. Angiotensin I-converting enzyme inhibitory peptides isolated from tofuyo fermented soybean food. *Biosci., Biotechnol., Biochem.* **2003**, *67*, 1278−1283.

(12) Rivas, M.; Garay, R. P.; Escanero, J. F.; Cia, P., Jr.; Cia, P.; Alda, J. O. Soy milk lowers blood pressure in men and women with mild to moderate essential hypertension. *J. Nutr.* **2002**, *132*, 1900−1902.

(13) Saiga, A.; Okumura, T.; Makihara, T.; Katsuta, S.; Shimizu, T.; Yamada, R.; Nishimura, T. Angiotensin I-converting enzyme inhibitory peptides in a hydrolyzed chicken breast muscle extract. *J. Agric. Food Chem.* **2003**, *51*, 1741−1745.

(14) Yoshii, H.; Tachi, N.; Ohba, R.; Sakamura, O.; Takeyama, H.; Itani, T. Antihypertensive effect of ACE inhibitory oligopeptides from chicken egg yolks. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* **2001**, *128*, 27−33.

(15) Matsui, T.; Li, C. H.; Tanaka, T.; Maki, T.; Osajima, Y.; Matsumoto, K. Depressor effect of wheat germ hydrolysate and its novel angiotensin I-converting enzyme inhibitory peptide, Ile-Val-Tyr, and the metabolism in rat and human plasma. *Biol. Pharm. Bull.* **2000**, *23*, 427−431.

(16) Suetsuna, K. Purification and identification of angiotensin I-converting enzyme inhibitors from the red alga *Porphyra yezoensis*. *J. Mar. Biotechnol.* **1998**, *6*, 163−167.

(17) Sato, M.; Hosokawa, T.; Yamaguchi, T.; Nakano, T.; Muramoto, K.; Kahara, T.; Funayama, K.; Kobayashi, A.; Nakano, T. Angiotensin I-converting enzyme inhibitory peptides derived from wakame (*Undaria pinnatifida*) and their antihypertensive effect in spontaneously hypertensive rats. *J. Agric. Food Chem.* **2002**, *50*, 6245−6252.

(18) Saito, Y.; Wanezaki, K.; Kawato, A.; Imayasu, S. Structure and activity of angiotensin I converting enzyme inhibitory peptides from sake and sake lees. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 1767−1771.

(19) Hsu, F. L.; Lin, Y. H.; Lee, M. H.; Lin, C. L.; Hou, W. C. Both dioscorin, the tuber storage protein of yam (*Dioscorea alata* cv. Tainong No. 1), and its peptic hydrolysates exhibited angiotensin converting enzyme inhibitory activities. *J. Agric. Food Chem.* **2002**, *50*, 6109−6113.

(20) Matsui, T.; Yukiyoshi, A.; Doi, S.; Sugimoto, H.; Yamada, H.; Matsumoto, K. Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. *J. Nutr. Biochem.* **2002**, *13*, 80−86.

(21) Li, C.H.; Matsui, T.; Matsumoto, K.; Yamasaki, R.; Kawasaki, T. Latent production of angiotensin I-converting enzyme inhibitors from buckwheat protein. *J. Pept. Sci.* **2002**, *8*, 267−274.

(22) Parrot, S.; Degraeve, P.; Curia, C.; Martial-Gros, A. In vitro study on digestion of peptides in Emmental cheese: analytical evaluation and influence on angiotensin I converting enzyme inhibitory peptides. *Nahrung* **2003**, *47*, 87−94.

(23) FitzGerald, R. J.; Meisel, H. Lactokinins: whey protein-derived ACE inhibitory peptides. *Nahrung* **1999**, *43*, 165−167.

(24) FitzGerald, R. J.; Meisel, H. Milk protein-derived peptide inhibitors of angiotensin-I-converting enzyme. *Br. J. Nutr.* **2000**, *84* (Suppl. 1), S33−S37.

(25) Fuglsang, A.; Rattray, F. P.; Nilsson, D.; Nyborg, N. C. Lactic acid bacteria: inhibition of angiotensin converting enzyme in vitro and in vivo. *Antonie Van Leeuwenhoek* **2003**, *83*, 27−34.

(26) Yamamoto, N.; Maeno, M.; Takano, T. Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *J. Dairy Sci.* **1999**, *82*, 1388−1393.

(27) Gilbert, C.; Blanc, B.; Frot-Coutaz J.; Portalier, R.; Atlan, D. Comparison of cell surface proteinase activities within the *Lactobacillus* genus. *J. Dairy Res.* **1997**, *64*, 561−571.

(28) Sasaki, M.; Bosman, B. W.; Tan, P. S. Comparison of proteolytic activities in various lactobacilli. *J. Dairy Res.* **1995**, *62*, 601−610.

(29) Juillard, V.; Laan, H.; Kunji, E. R.; Jeronimus-Stratingh, C. M.; Bruins, A. P.; Konings, W. N. The extracellular PI-type proteinase of *Lactococcus lactis* hydrolyzes β-casein into more than one hundred different oligopeptides. *J. Bacteriol.* **1995**, *177*, 3472−3478.

(30) Zevaco, C.; Gripon, J. C. Properties and specificity of a cell-wall proteinase from *Lactobacillus helveticus*. *Lait* **1988**, *68*, 393−408.

(31) Chabance, B.; Marteau, P.; Rambaud, J. C.; Migliore-Samour, D.; Boynard, M.; Perrotin, P.; Guillet, R.; Jolles, P.; Fiat, A. M. Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie* **1998**, *80*, 155−165.

(32) Roberts, P. R.; Burney, J. D.; Black, K. W.; Zaloga, G. P. Effect of chain length on absorption of biologically active peptides from the gastrointestinal tract. *Digestion* **1999**, *60*, 332−337.

(33) Fuglsang, A.; Nilsson, D.; Nyborg, N. C. Cardiovascular effects of fermented milk containing angiotensin-converting enzyme inhibitors evaluated in permanently catheterized, spontaneously hypertensive rats. *Appl. Environ. Microbiol.* **2002**, *68*, 3566−3569.

(34) Hata, Y.; Yamamoto, M.; Ohni, M.; Nakajima, K.; Nakamura, Y.; Takano, T. A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects. *Am. J. Clin. Nutr.* **1996**, *64*, 767−771.

(35) Fields, G. B.; Noble, R. L. Solid-phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Pept. Protein Res.* **1990**, *35*, 161−214.

(36) Yamamoto, N. Antihypertensive peptides derived from food proteins. *Biopolymers* **1997**, *43*, 129−134; **1997**, *43*, 401-402 (erratum).
(37) Binevski, P. V.; Sizova, E. A.; Pozdnev, V. F.; Kost, O. A. Evidence for the negative cooperativity of the two active sites within bovine somatic angiotensin-converting enzyme. *FEBS Lett.* **2003**, *550*, 84−88.
(38) Britton, J. R.; Kastin, A. J. Biologically active polypeptides in milk. *Am. J. Med. Sci.* **1991**, *301*, 124−132.
(39) Meisel, H.; Bockelmann, W. Bioactive peptides encrypted in milk proteins: proteolytic activation and thropho-functional properties [in process citation]. *Antonie Van Leeuwenhoek* **1999**, *76*, 207−215.
(40) Schlimme, E.; Meisel, H. Bioactive peptides derived from milk proteins. Structural, physiological and analytical aspects. *Nahrung* **1995**, *39*, 1−20.
(41) Smacchi, E.; Gobbetti, M. Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. *Food Microbiol.* **2000**, *17*, 129−141.
(42) Xu, R. J. Bioactive peptides in milk and their biological and health implications. *Food Rev. Int.* **1998**, *14*, 1−16.
(43) Minervini, F.; Algaron, F.; Rizzello, C. G.; Fox, P. F.; Monnet, 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*, 5297−5305.
(44) Nakamura, Y.; Masuda, O.; Takano, T. Decrease of tissue angiotensin I-converting enzyme activity upon feeding sour milk in spontaneously hypertensive rats. *Biosci., Biotechnol., Biochem.* **1996**, *60*, 488−489.
(45) Nakamura, Y.; Yamamoto, N.; Sakai, K.; Okubo, A.; Yamazaki, S.; Takano, T. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J. Dairy Sci.* **1995**, *78*, 777−783.
(46) Nakamura, Y.; Yamamoto, N.; Sakai, K.; Takano, T. Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme. *J. Dairy Sci.* **1995**, *78*, 1253−1257.
(47) Seppo, L.; Jauhiainen, T.; Poussa, T.; Korpela, R. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. *Am. J. Clin. Nutr.* **2003**, *77*, 326−330.
(48) 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.
(49) Yamamoto, N.; Akino, A.; Takano, T. Antihypertensive effects of different kinds of fermented milk in spontaneously hypertensive rats. *Biosci., Biotechnol., Biochem.* **1994**, *58*, 776−777.
(50) Pihlanto, L. A.; Rokka, T.; Korhonen, H. Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. *Int. Dairy J.* **1998**, *8*, 325−331.
(51) Kohmura, M.; Nio, N.; Kubo, K.; Minoshima, Y.; Munekata, E.; Ariyoshi, Y. Inhibition of angiotensin-converting enzyme by synthetic peptides of human β-casein. *Agric. Biol. Chem.* **1989**, *53*, 2107−2114.
(52) Maeno, M.; Yamamoto, N.; Takano, T. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. *J. Dairy Sci.* **1996**, *79*, 1316−1321.
(53) 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.

Received for review March 25, 2004. Revised manuscript received July 9, 2004. Accepted August 30, 2004.

JF049510T