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Angiotensin I-Converting-Enzyme-Inhibitory and Antibacterial Peptides from *Lactobacillus helveticus* PR4 Proteinase-Hydrolyzed Caseins of Milk from Six Species

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Sodium caseinates prepared from bovine, sheep, goat, pig, buffalo or human milk were hydrolyzed by a partially purified proteinase of *Lactobacillus helveticus* PR4. Peptides in each hydrolysate were fractionated by reversed-phase fast-protein liquid chromatography. The fractions which showed the highest angiotensin I-converting-enzyme (ACE)-inhibitory or antibacterial activity were sequenced by mass spectrum and Edman degradation analyses. Various ACE-inhibitory peptides were found in the hydrolysates: the bovine α_{S1}-casein (α_{S1}-CN) 24-47 fragment (f24-47), f169-193, and β-CN f58-76; ovine α_{S1}-CN f1-6 and α_{S2}-CN f182-185 and f186-188; caprine β-CN f58-65 and α_{S2}-CN f182-187; buffalo β-CN f58-66; and a mixture of three tripeptides originating from human β-CN. A mixture of peptides with a C-terminal sequence, Pro-Gly-Pro, was found in the most active fraction of the pig sodium caseinate hydrolysate. The highest ACE-inhibitory activity of some peptides corresponded to the concentration of the ACE inhibitor (*S*)-*N*-(1-[ethoxycarbonyl]-3-phenylpropyl)-ala-pro maleate (enalapril) of 49.253 μg/ml (100 μmol/liter). Several of the above sequences had features in common with other ACE-inhibitory peptides reported in the literature. The 50% inhibitory concentration (IC_{50}) of some of the crude peptide fractions was very low (16 to 100 μg/ml). Some identified peptides were chemically synthesized, and the ACE-inhibitory activity and IC_{50}s were confirmed. An antibacterial peptide corresponding to β-CN f184-210 was identified in human sodium caseinate hydrolysate. It showed a very large spectrum of inhibition against gram-positive and -negative bacteria, including species of potential clinical interest, such as *Enterococcus faecium*, *Bacillus megaterium*, *Escherichia coli*, *Listeria innocua*, *Salmonella* spp., *Yersinia enterocolitica*, and *Staphylococcus aureus*. The MIC for *E. coli* F19 was ca. 50 μg/ml. Once generated, the bioactive peptides were resistant to further degradation by proteinase of *L. helveticus* PR4 or by trypsin and chymotrypsin.

During the last decade, fundamental studies have opened a new field of research dealing with bioactive or biogenic substances derived from foods. Bioactive substances of food origin are considered to be dietary components which exert a regulatory activity in the human organism, beyond basic nutrition (30).

Milk naturally contains an array of bioactivities due to lysozyme, lactoferrin, immunoglobulins, growth factors, and hormones, which are secreted in their active form by the mammary gland (36). In addition, many bioactivities in milk are encrypted within the primary structure of milk proteins, requiring proteolysis for their release from precursors. Proteolysis may release these biogenic peptides during gastrointestinal transit or during food processing (for a review, see references 7, 20, and 30). These biological activities include opioid agonist and antagonist peptides, hypotensive peptides which inhibit angiotensin-I-converting enzyme (ACE), and mineral binding, immunomodulatory, antibacterial, and antithrombotic peptides (12, 13, 29). Generally, three strategies are used to identify and characterize biologically active peptides: (i) isolation from in vitro enzymatic digests of precursor proteins; (ii) isolation from in vivo gastrointestinal digests of precursor proteins; and (iii) chemical synthesis based on combinatorial library designs of peptides which have a structure identical to that of those known to be bioactive (4, 30).

Antihypertensive peptides inhibit ACE (peptidyl-dipeptide hydrolase; EC 3.4.15.1). ACE is a multifunctional ectoenzyme that is located in different tissues and plays a key physiological role in the renin-angiotensin, kallikrein-kinin, and immune systems. The enzyme is responsible for the increase in blood pressure by converting angiotensin-I to the potent vasoconstrictor, angiotensin-II, and by degrading bradykinin, a vasodilatory peptide, and enkephalins (34). After the discovery of competitive ACE inhibitors in snake venom (15), several ACE-inhibitory peptides were identified by in vitro enzymatic digestion of milk proteins or chemical synthesis of peptide analogues (20).

It is generally accepted that the total antibacterial effect in milk is greater than the sum of the individual contributions of immunoglobulin and nonimmunoglobulin defense proteins or peptides. This may be due to the synergistic activity of naturally occurring proteins and peptides, in addition to peptides gen-

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erated from inactive protein precursors (7). Antimicrobial peptides are observed throughout nature. In mammals, they are found both at the epithelial surfaces and within granules of phagocytic cells. They are an important component of innate defenses, since in addition to killing microorganisms, they are able to modulate inflammatory responses (10). Antimicrobial milk proteins, such as lactoferrin, its pepsin-derived peptide fragments (lactoferricins), casocidin-I, and isracidin were described in the early literature (5, 23, 46). More recently, antimicrobial and antifungal peptides were designed by using combinational libraries (4), and novel antibacterial peptides were expressed in vivo in *Escherichia coli* (44) or purified from a pepsin digest of human milk which corresponds to κ-casein (CN) f63-117 (24). Compared to the other potential functions of bioactive peptides, this remains to be further studied due to the interesting features of antimicrobial peptides.

Two other aspects deserve further consideration in this field: the role of lactic acid bacteria in generating bioactive peptides during food processing and the susceptibilities of milk from different species to serving as precursors of bioactive peptides.

Proteolytic activation of bioactive sequences by lactic acid bacteria has been debated recently due to the great advantage of using food-grade microorganisms to enrich foods with bioactive substances (20). The proteolytic system of lactic acid bacteria is very complex. It is composed of an extracellularly located serine proteinase, a transport system specific for di-, tri-, and oligopeptides, and a multitude of intracellular peptidases. Proteinases of lactic acid bacteria may hydrolyze more than 40% of the peptide bonds of α_{s1}- and β-CNs, producing oligopeptides of 4 to 40 amino acid residues (22). Several of the known bioactive peptides have been identified in dairy products. Consequently, lactic acid bacteria could potentially generate a large variety of peptides, including bioactive sequences, and the type of dairy product, the technology adopted, and, especially, strain selection, based on the specificity of proteolysis, are probably all factors that markedly influence the proteolytic activation of encrypted bioactive peptides.

CNs are the major proteins in the milk of most mammals. The primary sequences of the α_{s1}-, α_{s2}-, and β-CNs show considerable variation across species, consistent with rapidly evolving genes that are proposed to have a common precursor and with posttranslational modification. In contrast, the κ-CNs exhibit features that demonstrate also a separate origin among the species (18). To our knowledge, most of the studies on bioactive peptides have considered bovine milk proteins as precursors (20), and only a very few have used milk from different species (31).

The goal of this study was to use a proteinase from a food-grade microorganism (*Lactobacillus helveticus* PR4) to produce ACE-inhibitory and antimicrobial peptides from sodium caseinates of milk from six species (bovine, sheep, goat, pig, buffalo, and human) to show their potential to yield bioactive peptides. These peptides were isolated and sequenced, and their bioactivity was characterized; some were chemically synthesized.

MATERIALS AND METHODS

Substrates and chemicals. Hippuryl-L-histidil-L-leucine, ACE (from rabbit lung, lyophilized powder, ca. 3 U/mg of protein), rabbit lung acetone powder, furanacrylolyl tripeptide (Fa-Phe-Gly-Gly), enalapril, *p*-nitroanilides, trypsin (from bovine pancreas; ca. 10,000 U of *N*-α-benzoyl-L-arginine ethyl ester/mg of protein), *o*-phthaldialdehyde (OPA), chymotrypsin (from bovine pancreas; 40 to 60 U/mg of protein), insulin chain A, and other chemicals were from Sigma Chemical Co. (St. Louis, Mo.).

Microorganism and culture conditions. *L. helveticus* PR4, isolated from Italian cheeses and belonging to the culture collection of the Dipartimento di Protezione delle Piante e Microbiologia Applicata, University of Bari, Italy, was used. The bacterium was propagated in MRS broth (Oxoid Ltd, Basingstoke, United Kingdom) for 24 h at 37°C. Twenty-four-hour-old cells of *L. helveticus* PR4 were used to inoculate (3% [vol/vol]) 1 liter of MRS broth, which was incubated for 24 h at 37°C. After incubation, cells were harvested by centrifugation (10,000 × *g* for 10 min), washed in 50 mM Tris-HCl, pH 7.5, containing 0.1 M $CaCl_2$, and used for subcellular fractionation.

Proteinase purification. The cell wall lysate fraction was prepared by treatment with lysozyme in 50 mM Tris-HCl, pH 7.5, containing 24% (weight/vol) sucrose and 0.1 M $CaCl_2$, as described by Crow et al. (8). Cell wall lysate and loosely associated cell surface fractions, which both may contain cell wall-associated proteinase, were dialyzed against distilled water for 24 h at 4°C, pooled, freeze-dried, and used for proteinase purification.

To partially purify the cell wall-associated serine proteinase, samples (50 mg) of the above-described pooled fractions were applied to a Q-Sepharose HR 16/50 column (Amersham Biosciences, Uppsala, Sweden) that had been equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.1 M NaCl. Proteins were eluted at a flow rate of 0.3 ml/min with a linear NaCl gradient from 0.1 to 0.5 M. Fractions were assayed for proteinase activity by using fluorescein isothiocyanate-labeled casein (FITC casein) as the substrate (42). The most active fractions were pooled, dialyzed for 24 h at 4°C against 50 mM potassium phosphate buffer, pH 7.0, concentrated ca. 20-fold by freeze-drying, and resuspended in the same buffer. Lys-, Glu-, and Pro-*p*-nitroanilides were used as aminopeptidase substrates. The aminopeptidase activity was not detected on any of the three substrates, since no measurable amount of released *p*-nitroanilide was detected spectrophotometrically at 410 nm after 120 min of incubation at 37°C. The proteinase activity of the preparation was standardized by the method of Twinning (42) with FITC casein as substrate. The assay mixture contained 25 μl of 0.5 M FITC casein in 50 mM phosphate buffer, pH 7.0, and 75 μl of proteinase preparation; it was incubated for 2 h at 37°C. One unit of proteinase activity was the amount of enzyme that gave an increase of 0.1 U of fluorescence in 10 min at 37°C. Specific enzyme activity was expressed as the number of units per milligram of protein. Sodium caseinate hydrolysates were produced by using preparations which contained proteinase activity of 110 ± 4 U/mg.

The protein concentration was estimated by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, Calif.) using bovine serum albumin as the standard.

Sodium caseinate hydrolysates. Milk from six species, bovine (Frisona breed), sheep (Sarda breed), goat (Saanen breed), pig, buffalo (Bufalo Italiano type), and human, was used. Sodium caseinates were prepared by acidifying milk to pH 4.6 with HCl, heating to 35°C, and holding for 10 min; the precipitated casein was recovered by centrifugation at 5,000 × *g* for 10 min and washed with and suspended in distilled water. The pH of the casein suspension was adjusted to 7.0 with NaOH; when the acid casein was fully dissolved, the sodium caseinate was freeze-dried. Aliquots (1.5 g) of each sodium caseinate were redissolved in 30 ml of 50 mM phosphate buffer, pH 7.5, containing proteinase preparation and chloramphenicol (0.1 g/liter). After incubation at 37°C for 48 h with shaking (130 rpm), the enzyme reaction was stopped by freezing at −20°C.

Isolation of peptides from sodium caseinate hydrolysates. Peptides were separated from sodium caseinate hydrolysates by reversed-phase fast-protein liquid chromatography (RP-FPLC) using a Resource reversed-phase column and ÄKTA FPLC equipment with a UV detector operating at 214 nm (Amersham Biosciences). Aliquots (500 μl) of the sodium caseinate hydrolysates were diluted 1:4 with 0.05% (vol/vol) trifluoroacetic acid (TFA) and centrifuged at 12,000 × *g* for 10 min, and the supernatant was loaded onto the column. Elution was at a flow rate of 1 ml/min with a gradient (5 to 100%) of acetonitrile in 0.05% TFA. The concentration of CH_3CN was increased linearly from 5 to 46% between 16 and 62 min and from 46 to 100% between 62 and 72 min. Solvents were removed from the collected 2-ml fractions by freeze-drying. The fractions were redissolved in 500 μl of water and assayed for activities.

The peptide concentration in the sodium caseinate hydrolysates and related fractions was determined by the OPA method (6). The reaction mixture contained 500 μl of OPA reagent and 12.5 μl of peptide sample. Absorbance at 340 nm was determined. The peptide concentration was calculated from a standard curve prepared using tryptone (0.25 to 1.5 mg/ml) as a reference. The use of peptone as a standard gave similar results.

ACE activity and inhibition. ACE activity was determined by two methods. The first corresponded to a modified version of the method of Nakamura et al. (32). The hippuryl-L-histidil-L-leucine solution (100 μl) was mixed with 30 μl of a peptide fraction, a synthesized peptide, or water and 20 μl of ACE (100 mU/ml); the mixture was incubated for 60 min at 37°C. Synthesized peptides were used at the 50 percent inhibitory concentration (IC_{50}) (see below) concentration found for the crude fractions. When used in a mixture, the synthesized peptides were mixed in equal proportions by weight. The hippuric acid liberated by ACE was extracted with 0.85 ml of ethyl acetate. Controls without ACE added were included for each measurement (39).

The second method used to determine the ACE activity was that described by Vermeirssen et al. (43). This method was used since it was less expensive and more sensitive, as stated by the authors (38), and to check eventual drawback due to extraction of hippuric acid with ethyl acetate by the method of Nakamura et al. (32). The rabbit lung acetone extract was prepared by dissolving 1 g of rabbit lung acetone powder in 10 ml of 50 mM potassium phosphate buffer, pH 8.3, and ultracentrifuging the mixture for 40 min at 40,000 × *g*. The brown-orange supernatant had high ACE activity and was stored at 5°C (9). Prior to assay, the supernatant was diluted 10-fold with 50 mM potassium phosphate buffer, pH 8.3, so that it would have the same ACE activity as the commercial preparation (ca. 3 U/mg of protein) (Sigma Chemical Co.). Five hundred microliters of furanacrylolyl tripeptide (1 mM), dissolved in 50 mM Tris-HCl buffer (pH 8.3) containing 400 mM NaCl, and 300 μl of a peptide fraction, a synthesized peptide, or water were mixed and preincubated for 2 min at 37°C. After addition of 300 μl of the diluted rabbit lung acetone extract, the reaction mixture was incubated for 5 min at 37°C. Subsequently, the absorbance at 340 nm was measured over a time interval of exactly 20 min during incubation at 37°C. Percent inhibition was calculated based on a standard curve prepared from several dilutions of the rabbit lung acetone extract (43).

The ACE inhibitor (*S*)-*N*-(1-[ethoxycarbonyl]-3-phenylpropyl)-ala-pro maleate (enalapril) was used as a reference ACE-inhibitory substance at a range of concentrations from 49.253 to 0.049 μg/ml.

The concentration of an ACE-inhibitor (crude fraction) needed to inhibit 50% of ACE activity was defined as the IC_{50}. Due to some limitation of the OPA method for determining the peptide concentration, IC_{50} are considered "IC_{50} apparent values." Kinetic constants (K_i) for the inhibition of ACE activity by synthesized peptides were calculated from Dixon plots (11) and used to determine the IC_{50}.

Values of the percentage of ACE inhibition and IC_{50} are the average for three separated assays, and the coefficient of variation was always lower than 2%.

Antibacterial activity. A well-diffusion assay was used to detect the antibacterial activity of the peptide fraction (37). *L. helveticus* PR4, *Lactobacillus delbrueckii* subsp. *bulgaricus* B15, *Lactobacillus casei* subsp. *casei* 2047, *Enterococcus faecium* X95, and *Lactococcus lactis* subsp. *cremoris* ST7, isolated from cheeses and assayed on MRS and M17 agar media; *Lactobacillus plantarum* 20B and *Lactobacillus sanfranciscensis* CB1 from sourdough (sourdough bacterial agar medium), *Bacillus megaterium* F6 from fresh vegetables (LB agar medium); *Salmonella* spp. (Muller and Kauffman agar medium) and *Listeria innocua* DSM 20649 (LB agar medium) from fresh meat; and *Escherichia coli* F19 and K-12 (LB agar medium) and *Staphylococcus aureus* (M17 agar medium) of human origin were used as indicator strains. The assays were carried out in the different agar media overlaid with 15 ml of agar-H_2O (2% [wt/vol]) and 5 ml of different soft agar media which contained 10^4 CFU of an overnight culture of the indicator strain/ml. Wells, 2 mm in diameter, were cut into these agar plates, and 10 μl of the peptide fraction was placed into each well. Plates were stored at 4°C for 4 h to permit radial diffusion of the peptide, incubated at 28 or 37°C for 24 h, and subsequently examined for zones of inhibition. For a semiquantitative assay, a critical dilution assay was used (2).

Purification, sequencing and synthesis of ACE-inhibitory and antibacterial peptides. The fractions of the sodium caseinate hydrolysates with the highest ACE-inhibitory or antibacterial activities were rechromatographed by RP-FPLC on the Resource reversed-phase column. The centers of the inhibitory peaks were collected, freeze-dried, and used for sequencing.

The mass spectra of the peptides in the purified fractions were analyzed. Freeze-dried samples were dissolved in 10 μl of Milli-Q water, and 3 μl was deposited onto the plate with 1 μl of αCHCA matrix (5 mg of α-cyano-4-hydroxycinnamic acid/ml in 50:50 CH_3CN-TFA, 0.3% [vol/vol]). The analysis was performed with a matrix-assisted laser desorption ionization-time of flight mass spectrophotometer (Voyager DE-STR; Applied Biosystems, Palo-Alto, Calif.) with a laser at 337 nm and an acceleration voltage of 20,000 V. The amino acid sequences of the peptides were determined after derivatization of 5 μl of sample with phenylisothiocyanate and Edman degradation. These steps were carried out automatically on a 494A protein sequencer (Applied Biosystems). For some samples, the above analyses were completed by determination of the amino acid composition, which was performed on a Biotronik LC3000 analyzer (Bioritech, Chamarande, France) by ion exchange chromatography with a postcolumn derivatization by ninhydrin.

Some of the identified peptides were chemically synthesized by NeoSystem Laboratoire (Strasbourg, France). The purity of the synthesized peptides was greater than 92% as determined by high-performance liquid chromatography analysis and certified by the manufacturer.

Hydrolysis of synthesized peptides or crude peptide fractions by trypsin and chymotrypsin. Aliquots (10 μl) of the synthesized peptides (750 μM) or crude peptide fractions, at inhibitory concentrations (see Table 2), were incubated with 10 μl of trypsin or chymotrypsin (2 and 4 mg/ml, respectively) and 40 μl of 0.25 M Tris-HCl, pH 8.0, at 37°C for 50 min. The reaction was stopped with 100 μl of 0.1% TFA, and samples were analyzed by RP-FPLC as previously described. Insulin chain A (240 μg/ml) was used as the control, and the trypsin and chymotrypsin concentrations used were standardized to give about 80% hydrolysis of insulin chain A.

RESULTS

Production of sodium caseinate hydrolysates. The partially purified proteinase of *L. helveticus* PR4 was used to produce sodium caseinate hydrolysates based on the following considerations: (i) to use an enzyme from a food-grade bacterium which is widely used in cheese manufacture; (ii) to use an enzyme from a highly proteolytic lactic acid bacterium; and (iii) to use an enzyme which hydrolyzes caseins to oligopeptides, the size of which highly matches with the size of bioactive peptides. Sodium caseinates, and not whole milks, were subjected to proteolysis to have more precise information about protein precursors and to select milks from different species which will be used subsequently to produce fermented milks enriched with bioactive peptides.

The six sodium caseinate hydrolysates produced by the partially purified proteinase of *L. helveticus* PR4 had a peptide concentration of 0.515 (goat), 0.693 (human), 0.710 (sheep), 0.966 (pig), 1.238 (buffalo), and 1.812 mg/ml (bovine). Before fractionation by RP-FPLC, the above sodium caseinate hydrolysates showed ACE-inhibitory activities of 2 to 43%, and antibacterial activity against the indicator, *Escherichia coli* F19, was not found. The RP-FPLC peptide profiles of the sodium caseinate hydrolysates differed according to the milk species (Fig. 1). In particular, the hydrolysates of bovine and goat caseinates were rich in peptides in the hydrophobic and hydrophilic zones of the acetonitrile gradient, respectively. The other hydrolysates showed rather similar profiles. Prolonged incubation with the proteinase of *L. helveticus* PR4 did not modify the peptide profiles of the sodium caseinate hydrolysates (data not shown).

Isolation of ACE-inhibitory and antimicrobial peptides. Forty-one fractions of each sodium caseinate hydrolysate were collected by RP-FPLC. The ACE-inhibitory activity of each fraction was determined by the methods of Nakamura et al. (32) and of Vermeirssen et al. (43), who recently optimized and validated the method for the screening of bioactive peptides. The results obtained with the two methods did not differ. (*S*)-*N*-(1-[ethoxycarbonyl]-3-phenylpropyl)-ala-pro maleate (enalapril) was used as the standard under our experimental conditions; 70 to 100% of ACE inhibition by a peptide fraction corresponded to a concentration of the ACE inhibitor enalapril in the range of 4.925 to 49.253 μg/ml (10 to 100 μmol/liter).

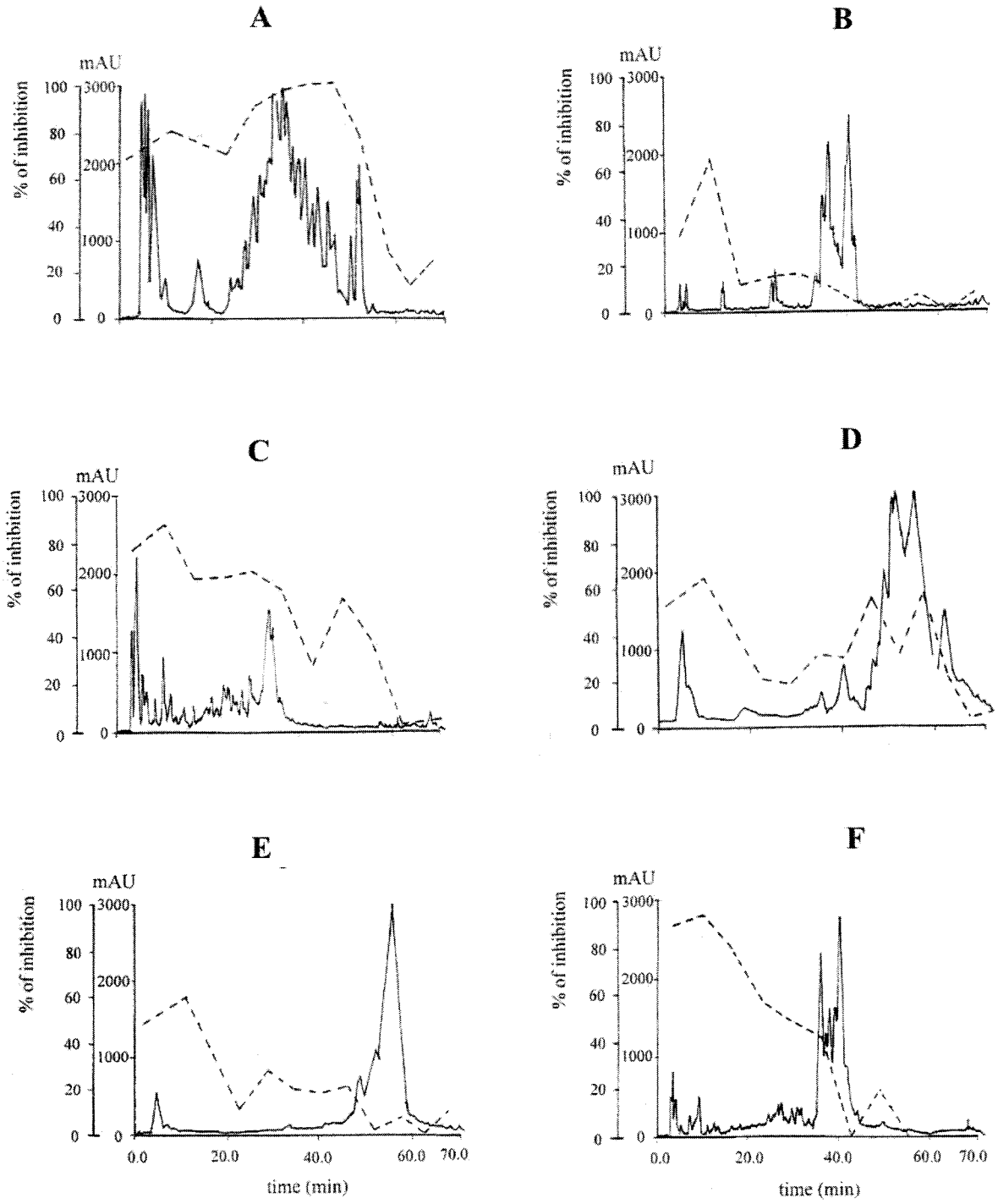

FIG. 1. RP-FPLC chromatograms of the sodium caseinate hydrolysates produced by a partially purified proteinase of *L. helveticus* PR4. (A) bovine; (B) sheep; (C) goat; (D) pig; (E) buffalo; and (F) human hydrolysates. The dashed line refers to the percentage of ACE inhibition.

The ACE-inhibitory indexes of the six sodium caseinate hydrolysates are shown in Fig. 1. All the hydrolysates contained fractions with a considerable ACE-inhibitory activity, which did not necessarily correspond to the largest peak areas. Several fractions from the bovine sodium caseinate hydrolysate showed 80 to 100% ACE inhibition, and some fractions from the goat and human sodium caseinate hydrolysates reached ca. 90%. Hydrolysates of sodium caseinate prepared from sheep, pig, and buffalo milk contained fractions which showed an ACE inhibition of ca. 70%.

All the peptide fractions of each sodium caseinate hydrolysate were assayed (10 μl) for antibacterial activity against *E. coli* F19 by a well diffusion assay. Under our experimental conditions, only fraction 19 of human sodium caseinate hydro-

TABLE 1. Sequences and corresponding CN fragments of peptides contained in crude fractions from sodium caseinate hydrolysates produced by a partially purified proteinase of *L. helveticus* PR4

Milk source or fraction	Sequence[a]	CN fragment	Calculated mass[b]	Expected mass[b]
Bovine				
16	LVYPFPGPIPNSLPQNIPP	β-CN f58-76	2,100.28	2,100.13
	FVAPFPEVFGKEKVNELSKDIGSE	α_{S1}-CN f24-47	2,194.35	2,194.14
	LGTQYTDAPSFSDIPNPIGSENSEK	α_{S1}-CN f169-193	2,122.27	2,121.93
17	LVYPFPGPIPNSLPQNIPP	β-CN f58-76	2,100.28	2,100.13
	FVAPFPEVFGKEKVNELSKDIGSE	α_{S1}-CN f24-47	2,194.35	2,194.14
18	LVYPFPGPIPNSLPQNIPP	β-CN f58-76	2,100.28	2,100.13
Sheep				
4	RPKHPI	α_{S1}-CN f1-6	746.90	746.46
	RPKH	α_{S1}-CN f1-4	537.32	537.35
	HPIKH	α_{S1}-CN f4-8	631.37	631.38
6	TVDQ	α_{S2}-CN f182-185	599.42	599.35
	HQK	α_{S2}-CN f186-188	411.46	411.22
Goat				
3	LVYPFPGP	β-CN f58-65	888.47	888.96
4	TVDQHQ	α_{S2}-CN f182-187	727.33	727.24
Buffalo				
4	LVYPFPGPI	β-CN f58-66	1,002.14	1,001.56
Human				
4	QPQ	β-CN f44-46	371.26	371.30
	VPQ	β-CN f77-79 or f137-139 or 155–157	342.26	342.22
	IPQ	β-CN f141-143 or f163-165 or κ-CN f74-76	356.36	356.39
19	QELLLNPTHQYPVTQPLAPVHNPISV[c]	β-CN f184-210	3,132.8	3,133.39

[a] Single-letter amino acid code is used.
[b] Monoisotopic masses are reported.
[c] The only peptide which had antibacterial activity. All the other peptides were ACE inhibitors.

lysate was found to be inhibitory for the growth of the indicator microorganism (data not shown).

Some of the ACE-inhibitory peptide fractions of each sodium caseinate hydrolysate which showed a value of ≥70% and the only antibacterial fraction from human milk were subsequently purified by RP-FPLC.

Sequencing and synthesis of peptides. Peptides in the purified fractions were subjected to mass spectrum analysis and Edman degradation. In some cases the analyses were completed by the determination of the amino acid composition. Most of the fractions contained a mixture of peptides, and the respective sequences are reported in Table 1.

Regarding peptide fractions which showed ACE-inhibitory activity, fractions 16, 17, and 18 of the bovine sodium caseinate hydrolysate contained the sequences β-CN f58-76, α_{S1}-CN f24-47, and α_{S1}-CN f169-193; β-CN f58-76 and α_{S1}-CN f24-47; and β-CN f58-76, respectively. In this case, as in some others, due to the very close positions within the acetonitrile gradient, an expected overlap of the sequences contained in the fractions was found. Mixtures of peptides originating at a unique protein zone were found in the sheep sodium caseinate hydrolysate: α_{S1}-CN f1-6, f1-4, and f4-8 in fraction 4 and α_{S2}-CN f182-185 and f186-188 in fraction 6. The two fractions purified from the goat sodium caseinate hydrolysate contained individual peptides: β-CN f58-65 in fraction 3 and α_{S2}-CN f182-187 in fraction 4. A peptide contained within the sequence 58-76 of β-CN, f58-66, was also found in the most ACE-inhibitory fraction purified from the buffalo sodium caseinate hydrolysate. The fraction of the human sodium caseinate hydrolysate contained a mixture of three tripeptides, which had a different residue at the N-terminal position and which potentially originated from various fragments of β- and κ-CN. A mixture of peptides with a C-terminal Pro-Gly-Pro sequence were found in fraction 3 of pig sodium caseinate hydrolysate, but the sequencing and mass spectrum analyses were not conclusive (data not shown).

The only antibacterial peptide fraction found in the human sodium caseinate hydrolysate contained the pure peptide β-CN f184-210.

Some peptides were chemically synthesized, since they had not previously been reported and are contained in a mixture with ACE-inhibitory activity, α_{S1}-CN f1-6, f1-4, and f4-8, and since they originated from a common sequence, β-CN f58-66, which, as a whole fragment or as part of a longer peptide, was found in several sodium caseinate hydrolysates.

Characterization of the ACE-inhibitory activity. The IC_{50} of the crude peptide fractions and of some synthesized peptides, alone or in mixtures, are shown in Table 2. The values of the apparent IC_{50} for crude peptide fractions may be overestimated due to the presence of free amino acids in the preparation, which interfered with the calculation of the peptide concentration, and, more generally, due to the possible breakdown of the large peptides as a result of the ACE activity. The three crude fractions from bovine sodium caseinate hydrolysates which had β-CN f58-76 in common showed a very low IC_{50} (16.2 to 57.2 μg/ml). When only a part (β-CN f58-66) of the above peptide was present, i.e., fractions 3 and 40 of the goat and buffalo sodium caseinate hydrolysates, the IC_{50} was slightly higher. Chemically synthesized β-CN f58-66 confirmed the ACE-inhibitory activity of the crude fraction. Crude peptide fraction 4 from sheep sodium caseinate hydrolysate, which contained a mixture of α_{S1}-CN f1-6, f1-4, and f4-8, had an IC_{50} of 120.2 mg/liter. The use of chemically synthesized peptides clearly showed that ACE inhibition was due mainly to the peptide α_{S1}-CN f1-6. The mixture of tripeptides in the crude

TABLE 2. ACE-inhibitory activities of crude peptide fractions and synthesized peptides

Crude peptide fraction or synthesized peptide	IC_{50}[a] (μg/ml or μmol/liter[b])
Bovine Na-caseinate hydrolysate	
Fraction 16	57.2
Fraction 17	16.2
Fraction 18 from bovine	23.9
Sheep sodium caseinate hydrolysate	
Fraction 4	120.2
Fraction 6	786.0
α_{S1}-CN f1-6 from fraction 4	30.1 (40.3[b])
α_{S1}-CN f1-4 from fraction 4	>1,000 (>1,863[b])
α_{S1}-CN f4-8 from fraction 4	800.5 (1,269.2[b])
α_{S1}-CN f1-6 + f1-4 from fraction 4	90.4
α_{S1}-CN f1-6 + f4-8 from fraction 4	70.2
α_{S1}-CN f1-4 + f4-8 from fraction 4	715.8
Goat sodium caseinate hydrolysate	
Fraction 3	147.3
Fraction 4	210.5
Buffalo Na-caseinate hydrolysate	
Fraction 40	112.6
β-CN f58-66 from fraction 40	180.6 (183.5[b])
Human sodium caseinate hydrolysate	
Fraction 4	228.1

[a] IC_{50} is the concentration of an ACE-inhibitor needed to inhibit 50% of ACE activity.
[b] IC_{50} was calculated in micromoles/liter for synthesized peptides only.

fractions of human sodium caseinate also showed a considerable ACE-inhibitory activity (IC_{50}, 228.1 μg/ml).

All the Dixon plots calculated for the synthesized peptides showed competitive inhibition (data not shown).

Characterization of antibacterial activity. As determined by the agar well-diffusion assay, β-CN f184-210, present in fraction 19 of the human sodium caseinate hydrolysate, inhibited the indicator microorganism, *E. coli* F19, at an estimated concentration of ca. 50 μg/ml. A wide spectrum of antibacterial activity, including gram-positive and -negative species, was found (Fig. 2 and Table 3). Except for *L. helveticus* and *L. plantarum* 20B, all the other species tested were strongly inhibited at a concentration of ca. 100 μg/ml. Inhibition also was observed against potentially pathogenic bacteria of clinical interest, such as *E. faecium*, *B. megaterium*, *E. coli* K-12, *L. innocua*, *Salmonella* spp., *Yersinia enterocolitica*, and *S. aureus*.

Hydrolysis of synthesized peptides or crude peptide fractions by trypsin and chymotrypsin. The synthesized peptides or the crude peptide fractions were treated with trypsin and chymotrypsin. Under assay conditions, which caused ca. 80% hydrolysis of insulin chain A, all the peptide preparations were resistant to hydrolysis (data not shown).

DISCUSSION

Posttranslational processing, alternative splicing of the gene product, or genetic polymorphism are responsible for a certain degree of CN heterogeneity across milk of different species (33). This heterogeneity is reflected in the relative proportions of particular CNs, identity of amino acids within CN sequences, and native conformation of individual CNs, which consequently influence the susceptibilities of CNs to enzymatic hydrolysis and to generation of bioactive peptides. In this study, the hydrolysis of sodium caseinates prepared from the milk of six species by a proteinase of *L. helveticus* PR4 produced various ACE-inhibitory and antimicrobial peptides.

β-CN f58-76, which was found alone or mixed with other peptides in fractions 16, 17, and 18 of the bovine sodium caseinate hydrolysate, showed the highest ACE-inhibitory activity (Tables 1 and 2). As shown for other ACE-inhibitory peptides (20), the three amino acids at the C terminus of β-CN f58-76 are hydrophobic, and 14 of the 19 amino acid residues in the peptide are nonpolar and hydrophobic. The fragment β-CN f58-72, containing the sequence of β-casomorphin-7, has been isolated from several cheeses and showed an antihypertensive effect and selective inhibition of bacterial peptidases (20). Other peptides, such as β-CN f57-64, f60-66, and f74-76,

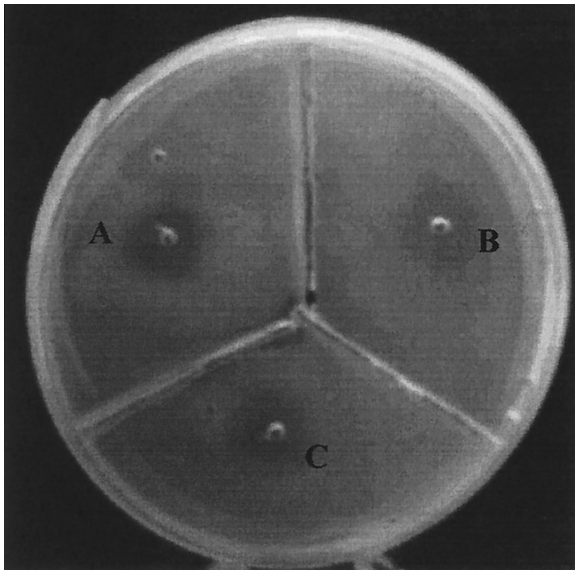

FIG. 2. Inhibition of *S. aureus* (A), *E. coli* K-12 (B), or *B. megaterium* (C) by fraction 19 of human sodium caseinate hydrolysate using the agar well diffusion assay.

TABLE 3. Antibacterial activity of β-CN f184-210 present in fraction 19 of the human sodium caseinate hydrolysate produced by a partially purified proteinase of *L. helveticus* PR4

Indicator species	Inhibition[a]
Lactobacillus helveticus PR4	−
Lactobacillus delbrueckii subsp. *bulgaricus* B15	+
Lactobacillus plantarum 20B	−
Lactobacillus casei subsp. *casei* 2047	+
Lactobacillus sanfranciscensis CB1	++
Enterococcus faecium X95	+
Lactococcus lactis subsp. *cremoris* ST7	++
Bacillus megaterium F6	+
Escherichia coli F19	++
Escherichia coli K-12	+
Listeria innocua DSM 20649	±
Salmonella spp.	+
Yersinia enterocolitica X8	+
Staphylococcus aureus ATCC 25923	±

[a] Symbols: ++, very large inhibition zone (ca. 2.0 cm); +, large inhibition zone (ca. 1.5 cm); ±, medium inhibition zone; −, no inhibition zone.

which originated from the sequence of β-CN f58-76, were shown to inhibit ACE, with IC_{50} ranging from 5 to 500 μmol/liter (17, 27, 28, 32, 45). The C-terminal amino acids of β-CN f58-76 coincide with the potent ACE inhibitor Ile-Pro-Pro, β-CN f74-76, identified in Calpis, a Japanase soft drink made from skim milk fermented with *L. helveticus* and *Saccharomyces cerevisiae* (32). The region of β-CN corresponding to f58-76 is considered to be a multifunctional sequence, since it contains different biological activities, such as antihypertensive, opioid agonist, immunomodulatory, and inhibitory activities, for proline endopeptidase (20). β-CN f58-76 also contains several cleavage sites for the proteinases of *L. helveticus* (45) and *L. delbrueckii* subsp. *bulgaricus* (19). Indeed, internal and shorter peptides, such as β-CN f58-65 and β-CN f58-66, were also found in fractions 3 and 4 of the goat and buffalo sodium caseinate hydrolysates, respectively (Table 1). Both of these peptides have hydrophobic amino acids, Gly-Pro-Ile or Trp-Gly-Pro, at the C terminus. A low concentration of β-CN f58-76 or related fragments in the other sodium caseinate hydrolysates cannot be excluded. However, the full sequence of β-CN f58-76 does not appear in the β-CNs of all the species considered; e.g., in human and pig β-CNs, 7 and 8 of the 19 amino acid residues differ from those of bovine, goat, and buffalo β-CN f58-76 (18).

The ACE-inhibitory crude fractions 16 and 17 of the bovine sodium caseinate hydrolysate contained α_{S1}-CN f24-47 also (Tables 1 and 2). Peptides from within this sequence, α_{S1}-CN f23-34, f23-37 (13, 17), f24-27, f25-27, f27-30, f28-34, and f32-34 (17, 26), have been produced from bovine casein enzymatic hydrolysis and showed bradykinin-potentiating activity on the uteri and ilea of rats. The IC_{50} of some of the above peptides varied largely, from 2 to >1,000 μmol/liter. Like α_{S1}-CN f24-47, not all of these peptides have hydrophobic residues at the C terminal end, but the whole sequences contain ca. 50% nonpolar hydrophobic amino acids. Alignment of the α_{S1}-CN f24-47 amino acid sequences shows that α_{S1}-CN from the milk of other species considered in this study differ from bovine α_{S1}-CN by at least five residues, the sequence of human milk being completely different (18).

α_{S1}-CN f169-193 was identified in fraction 16 of the bovine sodium caseinate hydrolysate, which contained β-CN f58-76 and α_{S1}-CN f24-47 also (Tables 1 and 2). α_{S1}-CN f169-199 and f194-199, produced by hydrolysis of bovine casein with trypsin (25) or the proteinase of *L. helveticus* (45), were shown to inhibit ACE. The apparent IC_{50} (ca. 24 μg/ml) for fraction 18, which contained β-CN f58-76 alone, or for fractions 16 and 17 (ca. 16 and 57 μg/ml), which contained a mixture of peptides, were of the same order of magnitude of those determined for in vivo-produced or -synthesized peptides which originated from related CN fragments (17, 28, 32, 45).

Fraction 4 of sheep sodium caseinate hydrolysate contained a mixture of peptides corresponding to α_{S1}-CN f1-6, f1-4, and f4-8 (Tables 1 and 2). These peptides were chemically synthesized and assayed for ACE-inhibitory activity. The results clearly showed that α_{S1}-CN f1-6 was the bioactive compound within the mixture, with an IC_{50} value of ca. 30.1 μg/ml, which compared well with the most active peptide identified from the same C-fraction (17). This peptide has not been reported previously as ACE inhibitory. It differs from α_{S1}-CN f1-4 and f4-8, especially due to the presence of hydrophobic Pro-Ile residues at the C-terminal end. A comparison of the sequence of α_{S1}-CN from the species considered in this study revealed less than 29% amino acid identity at the N terminus (18); only bovine and goat α_{S1}-CNs are completely identical with sheep α_{S1}-CN for the first six residues, but they differ for several subsequent amino acids. Fraction 6 of the sheep sodium caseinate hydrolysate had ACE-inhibitory activity also. It included α_{S2}-CN f182-185 and f186-188. A peptide originating from the same zone, α_{S2}-CN f182-187, was also found in fraction 4 of the goat sodium caseinate hydrolysate (Tables 1 and 2). α_{S2}-CNs are a very heterogeneous and evolutionarily divergent group of proteins. The sequence corresponding to α_{S2}-CN f182-187 is characteristic of the sheep and goat milks; none of the milks considered in this study show a homologous peptide fragment (18). ACE-inhibitory peptides corresponding to α_{S2}-CN f174-179 and f174-181 also were identified in a tryptic digest of bovine casein (41).

Fraction 4 of the human sodium caseinate hydrolysate contained a mixture of three ACE-inhibitory peptides (Tables 1 and 2) which may derive potentially from different zones of β-CN, f44-46, f77-79, f137-139, f155-157, f141-143, and f163-165. These fragments did not show identity with β-CNs of the other species.

Antimicrobial peptides from plants and animals manifest great structural diversity. Many peptides have α-helical structures. The majority of these peptides are cationic and amphipathic, but there are also hydrophobic α-helical peptides which possess antimicrobial activity (14). Some antimicrobial peptides are composed of 12 to 45 amino acids and have a net positive charge and a high content of hydrophobic residues (21). Although more-potent antibiotics are available, antimicrobial peptides show the advantages of being able to kill target cells rapidly and having a broad spectrum of activity, including activity for some of the more serious antibiotic-resistant pathogens in clinics. Since the rate of killing is higher than the rate of bacterial multiplication, this enhances the potential to overcome drug resistance. The main site of action of amphipathic or hydrophobic antimicrobial peptides is the cytoplasmic membrane, where they tend to assemble to form channels (3). Some known antimicrobial fragments from bo-

vine milk proteins are isracidin, α_{S1}-CN f16-38 (23), casocidin-I, α_{S2}-CN f165-203 (47), the casein-κ-derived glycomacropeptide, κ-CN f106-169 (1, 40), and lactoferricin (46). Cationic bovine α_{s2}-CN f183-207 and f164-179 obtained by pepsin digestion were shown to be antimicrobial against gram-positive and -negative bacteria (35). Depending on the target bacterial strains, these peptides exhibited MICs ranging from 8 to 95 μmol/liter. Fragment 183-207 had a consistently higher activity than f164-179, although both peptides showed a comparable hemolytic effect. Recently, a novel antimicrobial peptide, corresponding to human κ-CN f63-117, was generated by acidification and proteolysis with pepsin, which simulates digestion in the infant stomachs (24). An antimicrobial peptide, corresponding to β-CN f184-210, was produced by hydrolysis of the human sodium caseinate with the partially purified proteinase of *L. helveticus* PR4 (Table 1). It showed a very wide spectrum of activity against gram-positive and -negative bacteria (Table 3). By comparing some structural characteristics of this peptide with those of isracidin and lactoferricin (23, 46), the β-CN f184-210 has a very similar length (26 amino acids), a lower positive charge, a higher content of nonpolar hydrophobic residues (15 of the 26 amino acids), and some proline residues very near the C-terminal end of the peptide which could act to make its degradation by peptidases more difficult (44). β-CN f184-210 inhibited the indicator culture, *E. coli* F19, at a MIC of ca. 50 μg/ml, which compares well with the lethal concentrations, 4 to 10 μg/ml, of the more potent antimicrobial peptides (21). None of the other species considered in this study have CNs with a clear similarity to the C-terminal end of human β-CN (18).

Some regions in the primary structure of CNs are considered to be strategic, since they are partially protected from proteolytic breakdown (16). The profiles of the sodium caseinate hydrolysates did not change and the ACE-inhibitory indexes of the fractions were not changed after prolonged incubation with the proteinase of *L. helveticus* PR4. All the synthesized peptides and the crude fractions which contained ACE-inhibitory or antimicrobial peptides were resistant to in vitro hydrolysis by trypsin and chymotrypsin.

Over the past decade, Australia, North America, Europe, and Japan have seen a great increase in the consumption of functional foods (38). Although further research is needed, particularly in humans, to confirm the role of bioactive substances, certain dairy products can be considered physiological foods and precursors of several bioactive substances. This study shows the following: (i) the cell-wall associated serine proteinase of *L. helveticus* is a suitable enzyme for generation of bioactive peptides from CNs of different species; (ii) milks of different species all have the potential to yield bioactive peptides after enzymatic hydrolysis; (iii) in some cases, common but especially different bioactive peptides are generated from CNs from the different species, which is related to the level of sequence identity and native conformation of the CNs; (iv) sodium caseinate hydrolysates or related fermented milks may be considered as suitable functional foods, since the IC_{50} values of most of the ACE-inhibitory peptides found are compatible with the amounts (10 to 60 mg) of bioactive peptides potentially produced during proteolysis of 1 g of CN (26); and (v) human β-CN may be considered a precursor of a broad-spectrum antibacterial peptide.

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REFERENCES

1. **Aniansson, G., B. Andersson, R. Lindstedt, and C. Svanborg.** 1990. Antiadhesive activity of human casein against *Streptococcus termophilus* and *Haemophilus influenzae.* Microb. Pathog. **8:**315–323.
2. **Barefoot, S. F., and T. R. Klaenhammer.** 1983. Detection and activity of lactacin B, a bacteriocin produced by *Lactobacillus acidophilus.* Appl. Environ. Microbiol. **45:**1808–1815.
3. **Bechinger, B.** 1997. Structure and functions of channel-forming peptides: magainins, cecropins, melittin and alamethicin. J. Membr. Biol. **156:**197–211.
4. **Blondelle, S. E., and K. Lohner.** 2000. Combinatorial libraries: a tool to design antimicrobial and antifungal peptide analogues having lytic specificities for structure-activity relationship studies. Biopolymers **55:**74–87.
5. **Bullen, J. J., H. J. Rogers, and L. Leigh.** 1972. Iron binding proteins in milk and resistance to *E. coli* infections in infants. Brit. Med. J. **1:**69–75.
6. **Church, F. C., H. E. Swaisgood, D. H. Porter, and G. L. Catignani.** 1983. Spectrophotometric assay using *o*-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. J. Dairy Sci. **66:**1219–1227.
7. **Clare, D. A., and H. E. Swaisgood.** 2000. Bioactive milk peptides: a prospectus. J. Dairy Sci. **83:**1187–1195.
8. **Crow, V. L., R. Holland, and T. Coolbear.** 1993. Comparison of subcellular fractionation methods for *Lactococcus lactis* ssp. *lactis* and *Lactococcus lactis* ssp. *Cremoris.* Int. Dairy J. **3:**599–612.
9. **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.
10. **Devin, D. A., and R. E. W. Hancock.** 2002. Cationic peptides: distribution and mechanism of resistance. Curr. Pharmacol. Design **8:**703–714.
11. **Dixon, M.** 1953. The determination of enzyme inhibitor constants. Biochem. J. **55:**170–171.
12. **Dziuba, J., P. Mimkiewicz, and K. Plitnik.** 1996. Chicken meat proteins as potential precursors of bioactive peptides. Pol. J. Food Nutr. Sci. **4:**85–96.
13. **Dziuba, J., P. Minkiewicz, D. Nalecz, and A. Iwaniak.** 1999. Database of biologically active peptide sequences. Nahrung **43:**190–195.
14. **Epand, R. C., and H. J. Vogel.** 1999. Diversity of antimicrobial peptides and their mechanisms of action. Biochim. Biophys. Acta **1462:**11–28.
15. **Ferreira, S. H., D. C. Bartet, and L. J. Greene.** 1970. Isolation of bradykinin-potentiating peptides from *Bothrops jararaca* venom. Biochemistry **9:**2583–2593.
16. **Fiat, A. M., and P. Jollès.** 1989. Caseins of various origins and biologically active casein peptides and oligosaccharides: structural and physiological aspects. Mol. Cell. Biochem. **87:**5–30.
17. **FitzGerald, R. J., and H. Meisel.** 2000. Milk protein-derived inhibitors of angiotensin-I-converting enzyme. Brit. J. Nutr. **1:**S33–S37.
18. **Ginger, M. R., and M. R. Grigor.** 1999. Comparative aspects of milk caseins. Comp. Biochem. Physiol. B **124:**133–145.
19. **Gobbetti, M., P. Ferranti, E. Smacchi, F. Goffredi, and F. Addeo.** 2000. Production of angiotensin-I converting enzyme (ACE)-inhibitory peptides in fermented milks started by *Lactobacillus delbrueckii* subsp *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4. Appl. Environ. Microbiol. **66:**3898–3904.
20. **Gobbetti, M., L. Stepaniak, M. De Angelis, A. Corsetti, and R. Di Cagno.** 2002. Latent bioactive peptides in milk proteins: proteolytic activation and significance in dairy processing. Crit. Rev. Food Sci. Nutr. **42:**223–239.
21. **Hancock, R. E. W., and R. Leher.** 1998. Cationic peptides: a new source of antibiotics. Trends Biotechnol. **16:**82–88.
22. **Kunji, E. R. S., I. Mierau, A. Hagting, B. Poolman, and N. Konings.** 1996. The proteolytic system of lactic acid bacteria. Antonie Leeuwenhoek **70:**187–221.
23. **Lahov, E., and W. Regelson.** 1996. Antibacterial and immunostimulating casein-derived substances from milk: casecidin, isracidin peptides. Food Chem. Toxicol. **34:**131–145.
24. **Liepke, C., H. D. Zucht, W. G. Forsmann, and L. Standker.** 2001. Purification of novel peptide antibiotics from human milk. J. Chromatogr. **752:**369–377.
25. **Maruyama, S., K. Nakagomi, N. Tomizuka, and H. Suzuki.** 1985. Angiotensin I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. II. Isolation and bradykinin-potentiating activity on the uterus and the ileum of rats. Agric. Biol. Chem. **49:**1405–1409.
26. **Maruyama, S., H. Mitachi, H. Tanaka, N. Tomizuka, and H. Suzuki.** 1987. Studies on the active site and antihypertensive activity of angiotensin I-converting enzyme inhibitors derived from casein. Agric. Biol. Chem. **51:**1581–1586.
27. **Meisel, H.** 1993. Casokinins as inhibitors of angiotensin-converting-enzyme, p. 153–159. *In* G. Sawatzki and B. Renner (ed.), New perspectives in infant nutrition. Thieme, Stuttgart, N.Y.
28. **Meisel, H., and E. Schlimme.** 1994. Inhibitors of angiotensin-converting-enzyme derived from bovine casein (casokinins), p. 27–33. *In* V. Brantl and

H. Teschemacher (ed.), β-Casomorphins and related peptides: recent developments. Weinheim, VCH, New York, N.Y.
29. **Meisel, H., S. Günther, D. Martin, and E. Schlimme.** 1998. Apoptosis induced by modified ribonucleosides in human cell culture systems. FEBS Lett. **433:**265–268.
30. **Meisel, H.** 2001. Bioactive peptides from milk proteins: a perspective for consumers and producers. Austr. J. Dairy Technol. **56:**83–91.
31. **Mullally, M. M., H. Meisel, and R. J. FitzGerald.** 1996. Synthetic peptides corresponding to α-lactalbumin and β-lactoglobulin sequences with angiotensin-I-converting enzyme inhibitory activity. Biol. Chem. Hoppe-Seyler **377:**259–260.
32. **Nakamura, Y., N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki, and T. Takano.** 1995. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. J. Dairy Sci. **78:**777–783.
33. **Ng-Kwai Hang, K. F., and F. Grosclaude.** 1992. Genetic polymorphisms of milk proteins, p. 405–455. *In* P. F. Fox (ed.), Advanced Dairy Chemistry. Elsevier, London, United Kingdom.
34. **Petrillo, E. W., Jr., and M. A. Ondetti.** 1982. Angiotensin converting enzyme inhibitors: medicinal chemistry and biological actions. Med. Res. Rev. **2:**1–41.
35. **Recio, I., and S. Visser.** 1999. Identification of two distinct antibacterial domains within the sequence of bovine α_{s2}-casein. Biochim. Biophys. Acta **1428:**314–326.
36. **Schanbacher, F. L., R. S. Talhouk, and F. A. Murray.** 1997. Biology and origin of bioactive peptides in milk. Livest. Prod. Sci. **50:**105–123.
37. **Schillinger, U., and F. K. Lucke.** 1989. Antibacterial activity of *Lactobacillus sake* isolated from meat. Appl. Environ. Microbiol. **55:**1901–1906.
38. **Shah, N. P.** 2000. Effects of milk-derived bioactives: an overview. Brit. J. Nutr. **1:**S3–S10.
39. **Smacchi, E., and M. Gobbetti.** 1998. Peptides from several Italian cheeses inhibitory to proteolytic enzymes of lactic acid bacteria, Pseudomonas fluorescens ATCC 948 and to the angiotensin I-converting enzyme. Enzyme Microb. Technol. **22:**687–694.
40. **Stromqvist, M., P. Falk, S. Bergstrom, L. Hansson, B. Lonnerdal, S. Normark, and O. Hernell.** 1995. Human milk κ-casein and inhibition of *Helicobacter pylori* adhesion to human gastric mucosa. J. Pediatr. Gastroenterol. Nutr. **21:**288–296.
41. **Tauzin, J., L. Miclo, and J. L. Gaillard.** 2002. Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine αs2-casein. FEBS Lett. **531:**369–374.
42. **Twinning, S.** 1984. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. Anal. Biochem. **143:**30–34.
43. **Vermeirssen, V., J. Van Camp, and W. Verstraete.** 2002. Optimisation and validation of an angiotensin-converting enzyme inhibition assay for the screening of bioactive peptides. J. Biochem. Biophys. Methods **51:**75–87.
44. **Walker, J. R., J. R. Roth, and E. Altman.** 2001. An *in vivo* study of novel bioactive peptides that inhibit the growth of *Escherichia coli.* J. Peptide Res. **58:**380–388.
45. **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.
46. **Yamauchi, K., M. Tomita, T. J. Giehl, and R. T. Ellison.** 1993. Antibacterial activity of lactoferrin and a pepsin-derived lactoferrin peptide fragment. Infect. Immun. **61:**719–728.
47. **Zucht, H. D., M. Raida, K. Adermann, H. J. Magert, and W. G. Forssmann.** 1995. Casocidin-I: a casein-α_{s2} derived peptide exhibits antibacterial activity. FEBS Lett. **372:**185–188.