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Characterization of the Pattern of α_{s1}- and β-Casein Breakdown and Release of a Bioactive Peptide by a Cell Envelope Proteinase from *Lactobacillus delbrueckii* subsp. *lactis* CRL 581[▽]

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The cell envelope-associated proteinases (CEPs) of the lactobacilli have key roles in bacterial nutrition and contribute to the development of the organoleptic properties of fermented milk products as well, as they can release bioactive health-beneficial peptides from milk proteins. The influence of the peptide supply, carbohydrate source, and osmolites on the CEP activity of the cheese starter *Lactobacillus delbrueckii* subsp. *lactis* CRL 581 was investigated. The CEP activity levels were controlled by the peptide content of the growth medium. The maximum activity was observed in a basal minimal defined medium, whereas in the presence of Casitone, Casamino Acids, or yeast extract, the synthesis of CEP was inhibited 99-, 70-, and 68-fold, respectively. The addition of specific di- or tripeptides containing branched-chain amino acids, such as leucylleucine, prolylleucine, leucylglycylglycine, or leucylproline, to the growth medium negatively affected CEP activity, whereas dipeptides without branched-chain amino acids had no effect on the enzyme's production. The carbon source and osmolites did not affect CEP activity. The CEP of *L. delbrueckii* subsp. *lactis* CRL 581 exhibited a mixed-type $CEP_{I/III}$ variant caseinolytic specificity. Mass-spectrometric screening of the main peptide peaks isolated by reverse-phase high-pressure liquid chromatography allowed the identification of 33 and 32 peptides in the α_{s1}- and β-casein hydrolysates, respectively. By characterizing the peptide sequence in these hydrolysates, a pattern of α_{s1}- and β-casein breakdown was defined and is reported herein, this being the first report for a CEP of *L. delbrueckii* subsp. *lactis*. In this pattern, a series of potentially bioactive peptides (antihypertensive and phosphopeptides) which are encrypted within the precursor protein could be visualized.

Lactobacillus delbrueckii subsp. *lactis* is a homofermentative thermophilic lactic acid bacterium that is used as a starter culture for the manufacture of a variety of fermented dairy products, such as fermented sour milks and Swiss- and Italian-type cheeses. Like other lactic acid bacteria (LAB), *L. delbrueckii* subsp. *lactis* has a limited capacity to synthesize amino acids and is therefore dependent on the use of exogenous nitrogen sources for optimal growth (14, 16, 21, 32). As milk contains only small amounts of amino acids and short peptides (42), LAB depend on a complex proteolytic system to obtain essential amino acids from caseins during growth in milk. This specialized proteolytic system consists of a cell envelope-associated proteinase (CEP), transport systems to allow uptake of the resulting peptides, and several intracellular peptidases, which degrade peptides to amino acids (22, 36). The CEP is the key enzyme of this system as it is involved in the first step of casein degradation (22, 36). In addition to its vital role for bacterial growth in milk, CEP also contributes to the development of flavor and texture of fermented products (6, 26). Furthermore, certain CEPs can release bioactive health-beneficial peptides during milk fermentation (12, 13, 30).

The proteolytic system of lactococci is the best documented among LAB (10, 18, 22, 23, 28, 29, 31). The results of early experiments showed that in *Lactococcus lactis*, the synthesis of the cell wall proteinase PrtP during cell growth in peptide-rich medium is reduced compared to the rates of synthesis in milk or whey permeate medium with relatively low concentrations of peptides (18, 28). More recently, it has been demonstrated that the expression of several transcriptional units of the lactococcal proteolytic system, including *prtP*, was repressed in the presence of rich nitrogen sources, such as casein hydrolysates, Casitone, or Casamino Acids (9, 10, 35); the pleiotropic transcriptional regulator CodY was responsible for the repression of these proteolytic components (3, 11, 35).

CEPs of thermophilic lactobacilli have been isolated and characterized, mainly from *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus helveticus* (8, 27, 34, 37). The genes encoding proteinases from *L. delbrueckii* subsp. *bulgaricus* (8), *Lactobacillus rhamnosus* (33), *Lactobacillus acidophilus* (1), *L. helveticus* (34, 43), and *Lactobacillus paracasei* subsp. *paracasei* (17) have been identified. However, with the exception of PrtH of *L. helveticus* (14, 39), PrtB of *L. delbrueckii* subsp. *bulgaricus* (25), and PrtR of *L. rhamnosus* (33), no information is available about the regulation of proteinase production in lactobacilli. The synthesis of these enzymes was controlled by the peptide content of the growth medium (14, 33). The highest proteinase levels of *L. helveticus* CRL 1062 PrtH were found during cell growth in a simplified chemically defined medium (SCDM), while the synthesis of this enzyme was inhibited in

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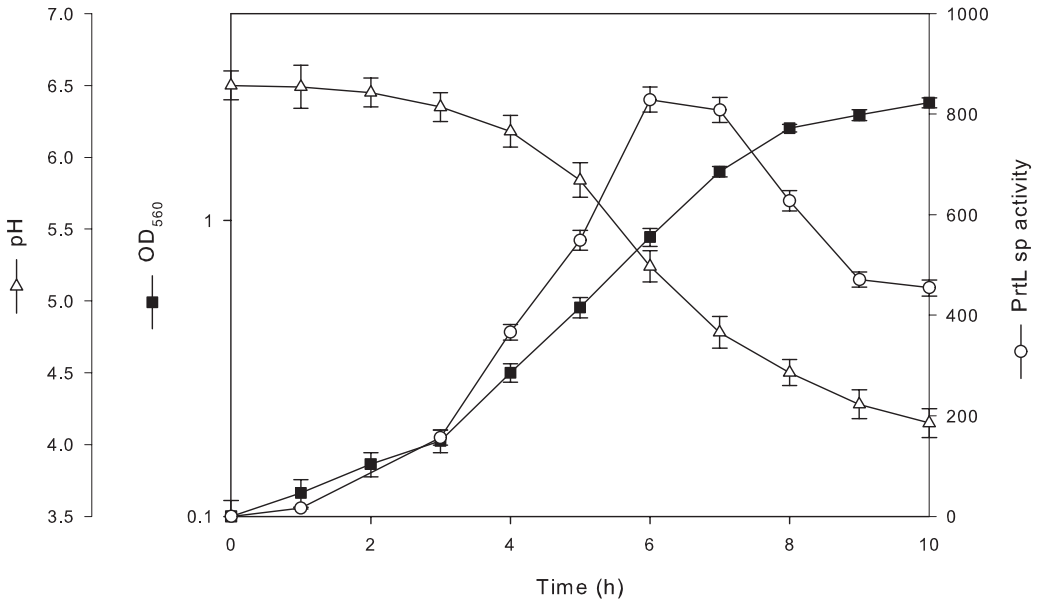

FIG. 1. Growth (OD_{560}), pH, and CEP specific activity of *L. delbrueckii* subsp. *lactis* CRL 581 grown in MDM. The LDH activity determined in the whole-cell suspensions was less than 1% of the total cell LDH activity, indicating that the proteolytic activity detected was due to the action of CEP. Error bars show standard deviations.

SCDM supplemented with Casitone, Casamino Acids, or the dipeptide leucylproline (LP) (14). Recently, array data identified genes of *L. helveticus* CNRZ 32 encoding cell envelope proteinases, oligopeptide transporters, and endopeptidases that were upregulated during growth in milk compared to their levels of expression in MRS medium (Biokar Diagnostics, France) (39).

L. delbrueckii subsp. *lactis* CRL 581, a thermophilic lactic acid bacterium isolated from Argentinian homemade hard cheese, synthesizes a CEP which has been partially characterized (15). Little information is available about the regulation of CEP biosynthesis in cells growing in the environmental conditions encountered in biomanufacturing and dairy products. In this sense, we studied the effects of various nitrogen sources, osmolites, and carbohydrates (lactose and its monosaccharide components) on the CEP of *L. delbrueckii* subsp. *lactis* CRL 581. Furthermore, we carried out a structural analysis of the peptides generated by this proteinase from α_{s1}- and β-caseins, tracing the pattern of its casein breakdown.

MATERIALS AND METHODS

Microorganisms, media, and growth conditions. *L. delbrueckii* subsp. *lactis* CRL 581 was isolated from a homemade Argentinian hard cheese and belongs to the culture collection of the Centro de Referencia para Lactobacilos (CERELA) of Argentina. In this study, a previously formulated minimal defined medium (MDM) containing 5 mM $CaCl_2$ was used (16). MDM (pH 6.5) was prepared from concentrated individual stock solutions, which were stored at 4°C after filtration, except for the cysteine solution that was freshly prepared. Medium and stock solutions were sterilized by filtration through a cellulose acetate membrane (0.20-μm pore size; Sartorius AG, Göttingen, Germany).

The low-molecular-mass (3,000 Da) peptides (LMMP) of Casitone (Difco Laboratories, Sparks, MD) were separated from the high-molecular-mass peptides (HMMP) by centrifugal filtration (3,000 × *g*) using filter units with a nominal molecular-mass limit of 3,000 Da (Centricon-3 concentrators; Amicon, Beverly, MA).

When indicated, MDM was supplemented with different nitrogen sources, such as 0.2 and 1% (wt/vol) Casitone (Difco), 0.2 and 1% (wt/vol) Casamino Acids (Difco), 1% (wt/vol) yeast extract (Difco), 0.3 mg/ml α_{s1}-casein (Sigma Chemical Co., St. Louis, MO), 0.3 mg/ml β-casein (Sigma), 1% (wt/vol) LMMP, 1% (wt/vol) HMMP, or the following di- and tripeptides (final concentrations, 1 and 5 mM; Sigma): glycylmethionine (GM), glycylproline (GP), glycyltyrosine, leucylleucine (LL), LP, prolylalanine (PA), prolylleucine (PL), tyrosylglycine, and leucylglycylglycine (LGG).

Working cultures of *L. delbrueckii* subsp. *lactis* were propagated in MRS broth (Biokar Diagnostics, France) at 40°C for 16 h. To eliminate carryover nutrients, the cells were harvested by centrifugation at 8,000 × *g* for 15 min, washed twice in sterile 50 mM sodium phosphate (pH 7.0), and resuspended in this buffer to the original volume. This cell suspension was used to inoculate the different media at an initial optical density (Cary 50; Varian, Inc., Australia) at 560 nm (OD_{560}) of 0.07. Bacterial growth was monitored by measuring the OD_{560}.

Water activity measurements. The water activity of culture medium containing 1% (wt/vol) lactose, glucose, or galactose; 5% (vol/vol) glycerol; or 0.25, 0.5, or 1% (wt/vol) NaCl was measured at 40°C by using a Thermoconstanter TH200 instrument with a TH temperature controller (0 to 50°C setting range), a BSK sensor, and an RTD-200 transmitter with a humidity range of water activity values from 0.05 to 1.00 (Novasina AG, Zürich, Switzerland). The humidity equilibrium was read until the measurements remained unchanged (after 48 h).

Proteinase activity assay. Cells grown in the different media were harvested by centrifugation (10,000 × *g* for 10 min at 4°C) at the exponential growth phase (OD_{560}, 0.90), washed twice with 0.85% (wt/vol) saline supplemented with 10 mM $CaCl_2$, and resuspended to a final OD_{560} of approximately 10 in 100 mM sodium phosphate buffer (pH 7.0). The proteinase activity of whole-cell suspensions was measured by a modification of the assay described previously (4) by the use of the chromogenic substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide (Sigma). The assay mixture, containing 214 μl of 50 mM sodium phosphate buffer (pH 7.0), 112 μl of 5 M NaCl (final concentration, 1.5 M), 19 μl of 20 mM substrate, and 30 μl of the cell suspension, was incubated at 40°C for 10 min. The reaction was stopped by adding 188 μl of 80% acetic acid, and the samples were centrifuged (10,000 × *g* for 5 min). The released nitroaniline was measured at 410 nm by using a VERSAmax microplate reader (Molecular Devices Corp., Sunnyvale, CA). One unit of proteinase was defined as the amount required to liberate 1 μmol of nitroaniline per minute. Specific activity was expressed as units of proteinase per mg protein. The protein concentration was determined by using a protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Richmond, CA). The level of cell lysis was determined by following the release of lactate dehydrogenase (LDH) with the method of Thomas (41).

Casein hydrolysis. Washed cells, harvested from the different media as indicated above, were suspended in 100 mM sodium phosphate buffer (pH 7.0). The

TABLE 1. CEP activity of *L. delbrueckii* subsp. *lactis* CRL 581 grown in MDM and MDM containing different peptide supplies

Peptide added to MDM (concn)	CEP sp act[a]	Inhibition factor[b]
None	829.0 ± 30	1.0
Casitone (1%)	8.4 ± 0.3	98.7
Casitone (0.2%)	16.3 ± 0.8	50.9
Casamino Acids (1%)	11.8 ± 5	70.3
Casamino Acids (0.2%)	41.5 ± 2.5	20.0
Yeast extract (1%)	12.2 ± 0.8	68.0
α_{s1}-Casein	820.6 ± 34	1.0
β-Casein	699.6 ± 25	1.2
All amino acids (10×)	825.5 ± 31	1.0
BCAA (50×)	498.2 ± 10	1.7

[a] Values are the means ± standard deviations of the results from three independent experiments. Specific activity is expressed as nmol · min · mg protein^{-1}. The lactate dehydrogenase activity was less than 0.06% of the total activity in each cell extract.
[b] Values are the ratios of the values obtained for MDM and MDM plus peptide supply.

suspensions were allowed to utilize the residual intracellular amino acids for 30 min at 40°C before casein degradation was carried out as described previously (14). Briefly, washed whole cells (OD_{560}, 10) were mixed with 5 mg/ml of substrate dissolved in 100 mM sodium phosphate (pH 7.0) at a ratio of 1:1. As substrate, α_{s1}-, β-, or κ-casein (Sigma) was used. These starting substrates were previously characterized by using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) to assess their purity. The resulting mixtures were incubated at 40°C. After various time intervals (0.25, 0.5, 1, 2, and 4 h), samples were centrifuged (10,000 × *g* for 10 min at 4°C) and the supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (24). Either Coomassie brilliant blue R-250 or silver staining (Bio-Rad) was used to visualize the proteins after SDS-PAGE.

LC-ESI-MS protein analysis. Liquid chromatography (LC) was performed by using a 2.1-mm-inner-diameter by 250-mm, C_{18}, 5-mm reverse-phase (RP) column (Vydac, Hesperia, CA) with a flow rate of 0.2 ml/min on an HP1100 modular system (Agilent, Palo Alto, CA). Solvent A was 0.1% trifluoroacetic acid (TFA) (vol/vol) in water; solvent B was 0.1% TFA (vol/vol) in acetonitrile.

TABLE 2. CEP activity of *L. delbrueckii* subsp. *lactis* CRL 581 grown in MDM supplemented with different peptides

Peptide added to basal MDM	CEP sp act[a]	Inhibition factor[b]
None	829.0 ± 30	1.0
1% LMMP	8.4 ± 0.3	98.7
1% HMMP	825 ± 28	1.0
1 mM LGG	125 ± 5	6.5
5 mM LGG	64 ± 3	12.9
1 mM LL	115 ± 5	7.2
5 mM LL	55 ± 3	15.1
1 mM LP	208 ± 9	4.0
5 mM LP	94 ± 4	8.8
1 mM PL	237 ± 11	3.5
5 mM PL	84 ± 4	9.9
5 mM GT	819 ± 25	1.0
5 mM PA	823 ± 31	1.0
5 mM TG	828 ± 28	1.0
5 mM GM	815 ± 30	1.0
5 mM GP	821 ± 27	1.0

[a] Values are the means ± standard deviations of the results from three independent experiments. Specific activity is expressed as nmol · min · mg protein^{-1}. The lactate dehydrogenase activity was less than 0.06% of the total activity in each cell extract.
[b] Values are the ratios of the values obtained for MDM and supplemented MDM.

TABLE 3. CEP activity of *L. delbrueckii* subsp. *lactis* CRL 581 grown in MDM and MDM supplemented with different sugar sources and osmolites

Supplement added to MDM (%)	CEP sp act[a]	Inhibition factor[b]	Water activity[c]
Glucose (1)	829.0 ± 30	1.0	0.976 ± 0.005
Galactose (1)	818.0 ± 35	1.0	0.976 ± 0.005
Lactose (1)	833.0 ± 29	1.0	0.976 ± 0.005
Glycerol (5)	618.6 ± 25	1.3	0.963 ± 0.005
NaCl (0.25)	609.6 ± 22	1.4	0.973 ± 0.005
NaCl (0.5)	590.2 ± 23	1.4	0.971 ± 0.005
NaCl (1)	609.6 ± 22	1.4	0.970 ± 0.005

[a] Values are the means ± standard deviations of the results from three independent experiments. Specific activity is expressed as nmol · min · mg protein^{-1}.
[b] Values are the ratios of the values obtained for MDM and supplemented MDM.
[c] Values are the means ± standard deviations of the results from three independent experiments.

The column was equilibrated at 5% solvent B. Separation of the peptides was effected with a gradient of 5% to 60% solvent B over 90 min. The column effluent was monitored by UV detection (220 nm), and each peak was manually collected. For high-pressure LC (HPLC) with positive ionization (electrospray ionization [ESI]) MS (LC-ESI-MS), the effluent from the column was injected on-line into a Platform (Micromass, England) MS equipped with a standard electrospray source via a 75-μm-inner-diameter fused-silica capillary. The mass spectra from 1,800 to 300 atomic mass units were scanned with a scan cycle of 5 s/scan. The source temperature was held at 180°C and the cone voltage at 40 V. The mass-scale calibration was obtained by using myoglobin as a reference compound.

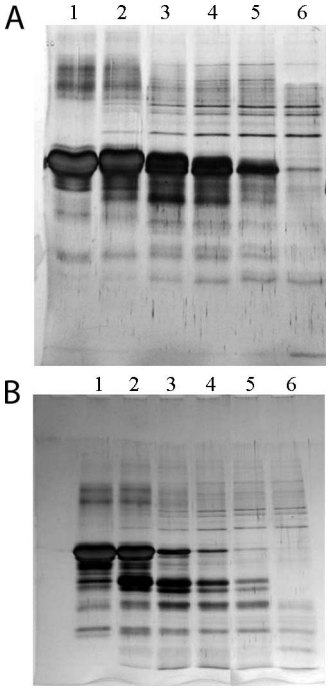

FIG. 2. Time course of hydrolysis of α_{s1}-casein (A) and β-casein (B) by *L. delbrueckii* subsp. *lactis* CRL 581 after growth in MDM medium. Purified α_{s1}-casein and β-casein were added to washed-cell suspensions of *L. delbrueckii* subsp. *lactis* CRL 581, and samples were taken immediately after addition (0 h, lanes 1) and at 15 min (lanes 2), 30 min (lanes 3), 1 h (lanes 4), 2 h (lanes 5), and 4 h (lanes 6) of incubation at 40°C. Cells were removed by centrifugation, and the supernatants were analyzed by SDS-PAGE.

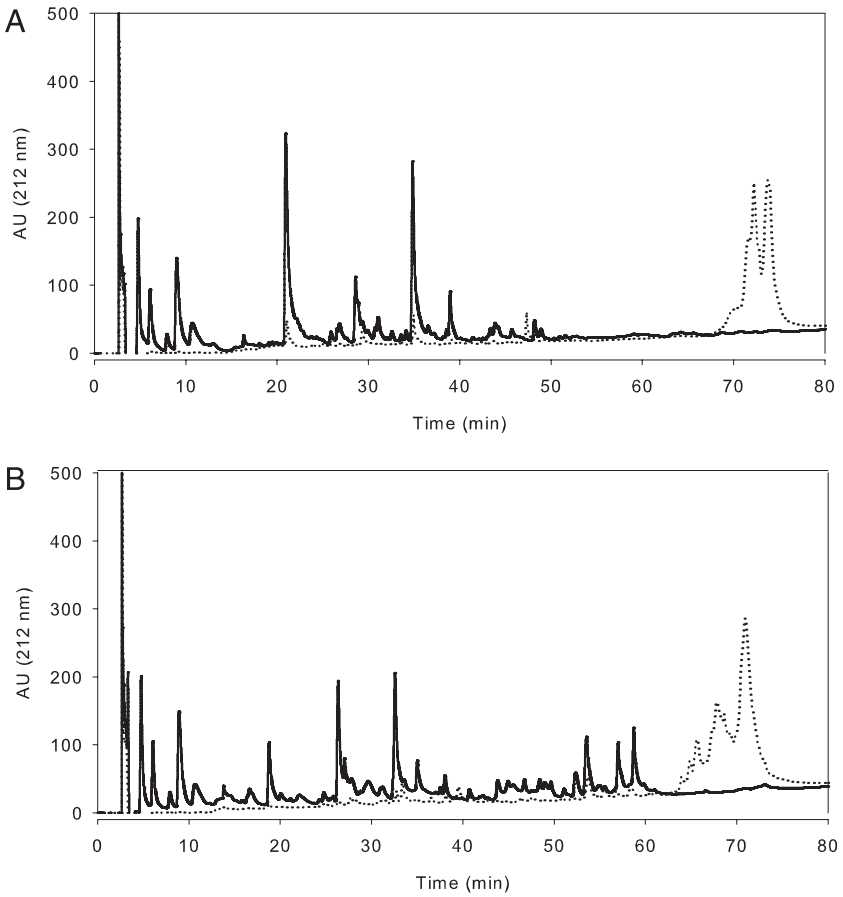

FIG. 3. RP-HPLC profiles of the peptides from the 1%-TFA-soluble fraction obtained from the control (0 h, dotted line) and after 4 h (solid line) of hydrolysis of α_{s1}-casein (A) and β-casein (B) by the action of CEP from *L. delbrueckii* subsp. *lactis* CRL 581. AU, absorbance units.

ESI-MS-MS. Tandem MS (MS-MS) data were obtained by using a Q-STAR MS (Applied Biosystems, Foster City, CA) equipped with nanospray interface (Protana, Odense, Denmark). Dried samples were resuspended in 0.1% TFA, desalted by using ZipTip C_{18} microcolumns (Millipore, Billerica, MA), and sprayed from gold-coated "medium-length" borosilicate capillaries (Protana). The capillary voltage used was 800 V. Double-charged ion isotopic clusters were selected by using the quadrupole mass filter and then induced to fragment by collision. The collision energy was 20 to 40 eV, depending on the size of the peptide. The collision-induced dissociation was processed by using Analyst 5 software (Applied Biosystems). The deconvoluted MS-MS spectrum was manually interpreted with the help of Analyst 5 software.

MALDI-TOF-MS analysis. MALDI-TOF-MS experiments were carried out on a PerSeptive Biosystems (Framingham, MA) Voyager DE-PRO instrument equipped with an N_2 laser (337-nm, 3-ns pulse width). Each spectrum was taken by using the following procedure: a 0.5-μl aliquot of the digest solution was loaded onto a stainless steel plate together with 0.5 μl of α-cyano-4-hydroxycinnamic acid matrix (1 g in 1 ml aqueous 50% acetonitrile). Mass spectrum acquisition was performed in both positive linear and reflectron mode by accumulating 200 laser pulses. The accelerating voltage was 20 kV. External mass calibration was performed with peptide mass standards (Sigma).

Peptide recognition. Signals recorded in the mass spectra were associated with the corresponding peptides based on the molecular weight by using PeptideTools software (Agilent).

RESULTS AND DISCUSSION

Influence of amino acids, peptides, and casein hydrolysates on CEP activity. The specific proteinase activity of *L. delbrueckii* subsp. *lactis* CRL 581 grown under non-pH-controlled conditions in basal MDM increased concomitantly with the cell growth until the middle of the exponential growth phase (OD_{560}, 0.96) and remained at high levels until the end of exponential growth (Fig. 1). Thereafter, at the beginning of the stationary growth phase, when the pH of the medium decreased below 4.8, the specific CEP activity declined sharply.

The results of early experiments showed that CEP activity levels in cells grown in the peptide-rich medium MRS were remarkably reduced compared with those found in a synthetic medium (104-fold lower), suggesting that the nitrogen source of the medium modulates the proteinase biosynthesis (15). To analyze the effect of the peptide supply on CEP, cells of *L. delbrueckii* subsp. *lactis* CRL 581 were grown in MDM containing different nitrogen sources, including Casitone, Casamino Acids, and yeast extract. Casitone is a pancreatic digest of casein consisting of small peptides and amino acids in a ratio of about 4:1 (28), whereas Casamino Acids is an acid casein hydrolysate in which free amino acids and small peptides are present in a ratio of 82% to 18%, respectively (according to the manufacturer). The CEP activities from CRL 581 cells grown in MDM with Casitone, Casamino Acids, and yeast extract were about 99-, 70-, and 68-fold lower, respectively, than that from cells grown in basal MDM (Table 1). The results of control experiments established that the peptide supply did not

have any direct inhibitory effect on the activity of this enzyme (data not shown). With a fivefold decrease in the concentration of Casitone or Casamino Acids in MDM, the CEP activity increased approximately 2 and 3.5 times, respectively (Table 1). The decrease of CEP production in cells grown in MDM supplemented with β-casein (ca. a 1.2-fold reduction) was less marked than that observed in cells grown in MDM with Casitone or Casamino Acids, whereas α_{s1}-casein had no significant effect on CEP biosynthesis (Table 1). The influence of α_{s1}- and β-casein hydrolysates, obtained by the action of CRL 581 proteinase, on CEP synthesis was similar to that observed with whole α_{s1}- and β-caseins, respectively (data not shown). These data suggest that the hydrolysis of the main milk proteins by CEP of *L. delbrueckii* subsp. *lactis* CRL 581 might not greatly affect its production during cell growth.

To determine if certain peptides are involved in the regulation of CEP biosynthesis, eight specific dipeptides, one tripeptide, and two peptide fractions (LMMP and HMMP) from Casitone were evaluated. The CEP activity levels of *L. delbrueckii* subsp. *lactis* CRL 581 grown in MDM supplemented with LMMP were similar to those obtained in cells grown in MDM supplemented with Casitone (99-fold reduction; Table 2). The addition of LGG, LL, LP, or PL (final concentration, 1 mM) to MDM led to a 6.5-, 7-, 4-, or 3.5-fold reduction in CEP activity, respectively (Table 2). An increase of up to 5 mM in the concentration of these dipeptides resulted in a further two- or threefold reduction of CEP activity compared to the activity obtained in the presence of 1 mM of the peptide mentioned above. LGG, LL, LP, and PL contain leucine as a branched-chain amino acid (BCAA). In contrast, no effect on CEP activity was observed by the supplementation of MDM with 1 to 5 mM of GT, PA, TG, GM, GP (dipeptides without BCAA), or HMMP (Table 2). No inhibitory effect on proteinase activity from the presence of high concentrations (10-fold increase) of each of the 20 amino acids in the growth medium was observed. However, a 50-fold increase in BCAA concentration in MDM led to a repression of proteinase synthesis of 40% (Table 1). *L. delbrueckii* subsp. *lactis* is auxotrophic for BCAA (16). The observation that a 50-fold excess of BCAA had only a 1.7-fold repression effect in CEP production might be explained as the result of a low efficiency of amino acid uptake that would limit BCAA availability inside the cell (9).

The results obtained indicated that the regulation of the biosynthesis of CEP of *L. delbrueckii* subsp. *lactis* is similar to that of other LAB, including *L. lactis* (10, 22, 31), *L. helveticus* (14, 39), *L. delbrueckii* subsp. *bulgaricus* (25), and *L. rhamnosus* (33). However, the strength of repression may be different (15). In *L. lactis*, the expression of seven transcriptional units of the proteolytic system, including *prtP*, *prtM*, *opp-pepO1*, *pepD*, *pepN*, *pepC*, and *pepX*, was shown to be repressed 5- to 150-fold upon the addition of Casitone to the growth medium, and it was controlled negatively by specific dipeptides containing BCAA (9, 10). In *L. helveticus* CNRZ 32, tiled microarrays identified genes encoding cell-envelope proteinases, oligopeptide transporters, and endopeptidases that were upregulated in milk-grown culture compared to their levels of expression in a peptide-rich MRS medium (39).

Effects of carbon source and osmolites on proteinase activity. The influence of the carbon source on CEP synthesis was tested after cell growth in MDM containing glucose, galactose,

TABLE 4. Peptides identified in the α_{s1}- and β-casein hydrolysates produced by CEP activity of *L.delbrueckii* subsp. *lactis* CRL 581

Mass (Da)		Peptide identity[a]
Measured	Expected	
1,139.67	1,139.67	α_{s1}-Casein (1–9)
543.27	542.27	α_{s1}-Casein (10–14)
943.01	942.50	α_{s1}-Casein (14–21)
1,245.67	1,245.67	α_{s1}-Casein (14–23)
474.30	473.25	α_{s1}-Casein (15–18)
5,595.01	5,595.31	α_{s1}-Casein (52–96) 5P
1,048.01	1,047.48	α_{s1}-Casein (75–82) 1P
803.38	802.37	α_{s1}-Casein (83–89)
959.48	958.47	α_{s1}-Casein (83–90)
1,122.40	1,121.54	α_{s1}-Casein (83–91)
1,234.62	1,234.62	α_{s1}-Casein (83–92)
1,292.64	1,291.64	α_{s1}-Casein (83–93)
675.20	674.28	α_{s1}-Casein (84–89)
831.38	830.38	α_{s1}-Casein (84–90)
994.40	993.44	α_{s1}-Casein (84–91)
1,106.57	1,106.52	α_{s1}-Casein (84–92)
546.23	545.23	α_{s1}-Casein (85–89)
702.30	701.33	α_{s1}-Casein (85–90)
865.30	864.3	α_{s1}-Casein (85–91)
571.30	570.3	α_{s1}-Casein (110–114)
1,473.64	1,472.67	α_{s1}-Casein (110–121) 1P
921.40	920.38	α_{s1}-Casein (115–121) 1P
1,239.04	1,138.45	α_{s1}-Casein (115–123) 1P
454.19	453.22	α_{s1}-Casein (125–128)
661.01	660.33	α_{s1}-Casein (135–140)
790.60	789.37	α_{s1}-Casein (135–141)
717.30	716.30	α_{s1}-Casein (144–148)
389.19	388.16	α_{s1}-Casein (175–178)
1,028.44	1,027.48	α_{s1}-Casein (180–189)
941.01	940.45	α_{s1}-Casein (181–189)
825.42	825.42	α_{s1}-Casein (182–189)
713.31	712.34	α_{s1}-Casein (183–189)
546.20	545.27	α_{s1}-Casein (196–199)
1,496.46	1,495.79	β-Casein (1–13)
3,078.53	3,078.34	β-Casein (16–39) 3P
1,461.56	1,460.58	β-Casein (32–42) 1P
1,576.60	1,575.31	β-Casein (32–43) 1P
1,946.74	1,945.79	β-Casein (32–46) 1P
2,684.01	2,683.19	β-Casein (32–52) 1P
3,111.30	3,111.39	β-Casein (32–56) 1P
1,247.52	1,246.53	β-Casein (37–46)
1,983.92	1,983.93	β-Casein (37–52)
1,855.31	1,854.89	β-Casein (38–52)
755.16	754.41	β-Casein (47–52)
3,018.46	3,018.59	β-Casein (53–80) var A2
2,590.38	2,590.39	β-Casein (57–80) var A2
326.20	325.20	β-Casein (74–76)
439.26	438.28	β-Casein (74–77)
545.26	544.29	β-Casein (78–82)
459.25	458.25	β-Casein (84–87)
976.43	975.51	β-Casein (111–118)
677.30	676.33	β-Casein (126–131)
1,014.48	1,013.53	β-Casein (146–154)
488.42	487.24	β-Casein (157–160)
575.29	574.28	β-Casein (157–161)
1,783.01	1,781.05	β-Casein (161–177)
1,667.90	1,667.90	β-Casein (192–206)
1,554.82	1,554.82	β-Casein (193–206)
1,148.25	1,147.63	β-Casein (194–204)
1,392.73	1,391.76	β-Casein (194–206)
1,020.23	1,019.58	β-Casein (195–204)
1,264.64	1,263.69	β-Casein (195–206)
1,037.60	1,037.60	β-Casein (197–206)
939.51	938.53	β-Casein (198–206)
826.44	825.45	β-Casein (199–206)

[a] Numbers refer to amino acid positions in the α_{s1} = or β-casein protein sequence. P, phosphate group; var A2, β-casein variant A2.

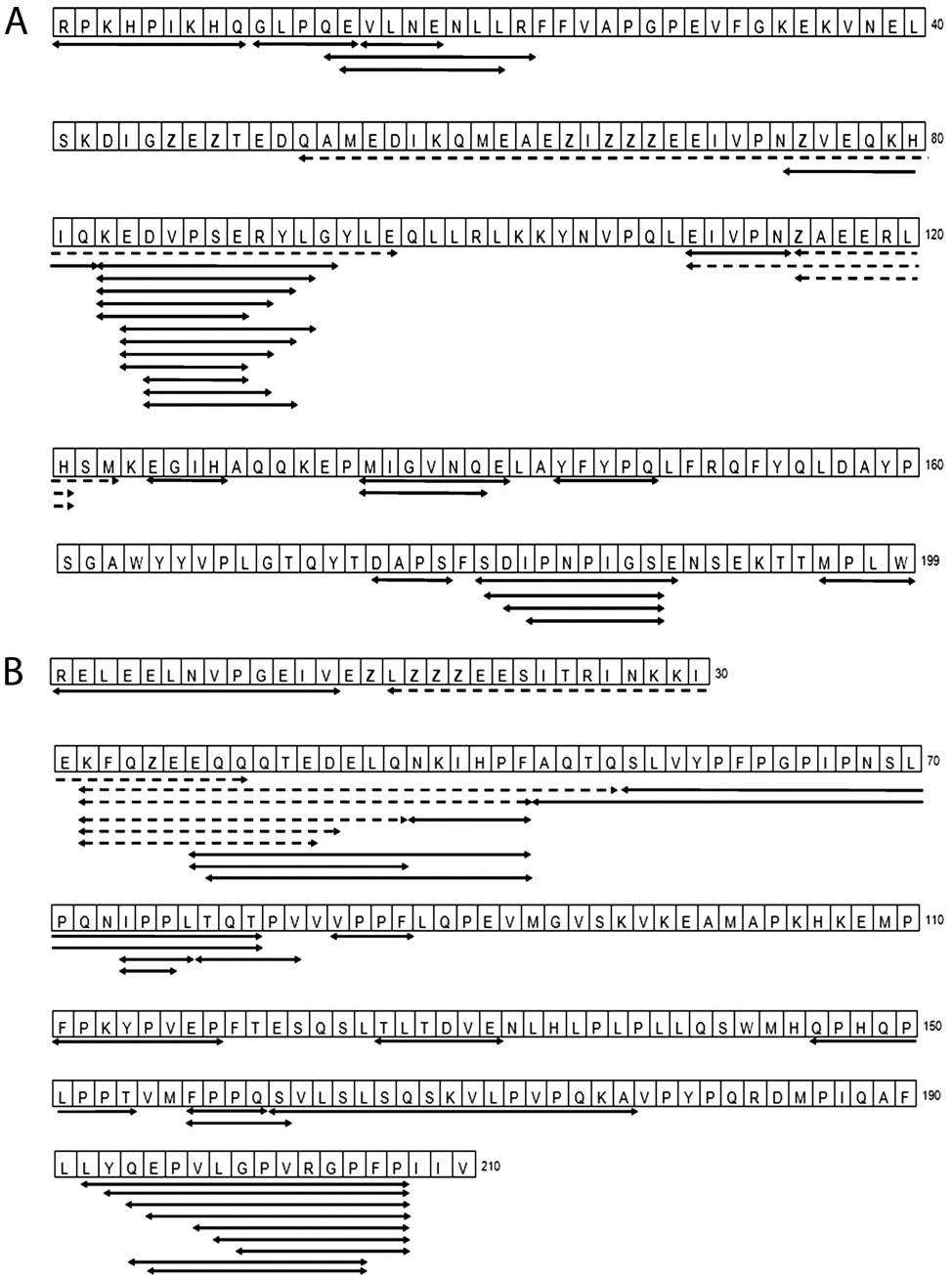

FIG. 4. Locations of the main peptides (double-ended arrows) identified in the primary sequences of α_{s1}-casein (A) and β-casein (B) and released by CEP from *L. delbrueckii* subsp. *lactis* CRL 581. Zs are phosphoserines. The phosphopeptides are indicated with dashed-line double-ended arrows.

or lactose. Cell cultures of *L. delbrueckii* subsp. *lactis* CRL 581 grown in MDM containing lactose or glucose displayed similar growth rates (μ, 0.35 h^{-1}), while cell growth on galactose was limited (μ, 0.12 h^{-1}), with a prolonged lag phase. However, the proteinase specific activity values were independent of the carbon source (Table 3).

Considering that lowering water activity in food by adding salt is an important step in cheese ripening, the effects of osmolites, such as NaCl or glycerol, on proteinase regulation were evaluated (Table 3). For that purpose, cells were grown in MDM containing different concentrations of NaCl (0.25, 0.5, 1, and 2%) or glycerol (5, 10, and 15%). The addition of up to 1% NaCl or 5% glycerol reduced CEP activity slightly (about 1.4-fold, Table 3). At higher concentrations of these osmolites (>1% NaCl or >5% glycerol), the metabolic activity of the cell (lactic acid production) and the growth rate decreased considerably and did not allow CEP activity to be determined (data not shown).

Casein hydrolysis and cleavage-site specificity. The ability of *L. delbrueckii* subsp. *lactis* CRL 581 to hydrolyze α_{s1}-, β-, and κ-caseins was tested after cell growth in MDM. Degradation of α_{s1}- and β-caseins was already observed in the first 15 min of incubation, and they were completely digested after 4 h (Fig.

2). During the course of the reactions (from 0 to 4 h), the relative amounts of the cleavage products of α_{s1}- and β-caseins changed significantly, as several degradation bands could be visualized (Fig. 2). Contrariwise, κ-casein was not hydrolyzed by *L. delbrueckii* subsp. *lactis* CRL 581 CEP (data not shown). Therefore, according to the substrate specificity, this proteinase has a caseinolytic activity comparable to that of the P_{III}-type lactococcal proteinases (5, 22). This caseinolytic specificity of CRL 581 CEP differed from that observed for *L. delbrueckii* subsp. *lactis* ACA-DC 178, which hydrolyzed β-casein predominantly and α_{s1}- and κ-caseins at much lower rates (43). The hydrolysis of α_{s1}- and β-caseins by the CEP of *L. delbrueckii* subsp. *lactis* CRL 581 after 4 h of incubation at 40°C was analyzed by RP-HPLC (Fig. 3). The main RP-HPLC peaks were collected and identified by MS analysis. Thirty-three and 32 peptides were identified in the α_{s1}- and β-casein hydrolysates, respectively (Table 4 and Fig. 4). The α_{s1}-casein hydrolysate contained peptides with from 4 to 44 amino acids (388.16 to 5,595.31 Da), including five phosphorylated peptides (Gln_{52} to Glu_{96}, Ser_{75} to Gln_{82}, Glu_{110} to His_{121}, Ser_{115} to His_{121}, and Ser_{115} to Met_{123}). This is considerably more than the 2 and 10 peptides which have been previously described for *L. helveticus* CNRZ 303 (45) and *L. helveticus* CP790 (44), respectively, the only α_{s1}-casein hydrolysates identified so far for LAB. The cleavage sites detected for the CEP of *L. delbrueckii* subsp. *lactis* CRL 581 were different from those described for the CEP of *L. helveticus* (Fig. 5). The *L. delbrueckii* subsp. *lactis* CRL 581 CEP prefers a glutamine (Q) or glutamic acid (E) side chain on the residue whose carbonyl carbon is part of the peptide bond to be cleaved; approximately 35% of the released peptides from α_{s1}-casein had Q or E at the C-terminal part of the molecule (Fig. 4A).

The α_{s1}-casein (f1-23) fragment, used for determining the proteinase specificity in *Lactococcus*, was hydrolyzed at least at the Gln_9-Gly_{10}, Gln_{13}-Glu_{14}, Glu_{14}-Val_{15}, Glu_{18}-Asn_{19}, and Leu_{21}-Arg_{22} bonds (Table 4 and Fig. 5). The lactococcal CEPs are classified in seven groups (from a to g) according to their specificities toward the α_{s1}-casein fragment comprising residues 1 to 23 (5). Like CEP of *L. delbrueckii* subsp. *lactis* CRL 581, many CEPs of LAB are able to hydrolyze the Gln_9-Gly_{10}, Gln_{13}-Glu_{14}, and Leu_{21}-Arg_{22} bonds (Fig. 5). To our knowledge, the Glu_{14}-Val_{15} and Glu_{18}-Asn_{19} cleavage sites are specific for *L. delbrueckii* subsp. *lactis* CRL 581 and have never been identified for any of the previously described CEPs of LAB (Fig. 5). Therefore, the specificity of the *L. delbrueckii* subsp. *lactis* CRL 581 CEP toward the α_{s1}-casein (f1-23) fragment did not fit into the criteria for one of the groups distinguished so far among the lactococcal CEP variants (5), and the *L. delbrueckii* subsp. *lactis* CRL 581 CEP could be classified as a mixed-type $CEP_{I/III}$ variant (2).

The β-casein hydrolysate contained 32 peptides of from 3 to 27 amino acids (325.20 to 3,018.59 Da), of which six were phosphopeptides: Leu_{16} to Gln_{39}, Lys_{32} to Glu_{42}, Lys_{32} to Asp_{43}, Lys_{32} to Gln_{46}, Lys_{32} to Phe_{52}, and Lys_{32} to Gln_{56} (Table 4 and Fig. 4B). The identification of peptides produced during β-casein hydrolysis by lactobacilli has been described for *L. delbrueckii* subsp. *lactis* ACA-DC 178 and *L. helveticus* CNRZ 303 and CP790, for which 4, 6, and 15 peptides, respectively, were identified (43, 44, 45). Four bonds (Ser_{15}-Leu_{16}, Glu_{42}-Asp_{43}, Leu_{192}-Tyr_{193}, and Pro_{206}-Ile_{207}) were cleaved by both *L. delbrueckii* subsp. *lactis* CRL 581 and *L. helveticus* CP790, while three bonds (Met_{156}-Phe_{157}, Ser_{161}-Val_{162}, and Leu_{192}-Tyr_{193}) were cleaved by the CEPs of both *L. delbrueckii* subsp. *lactis* CRL 581 and ACA-DC 178. All the other cleavage sites observed in this study were different from those reported for other lactobacillus proteinases. In addition, unlike the proteinase of *L. delbrueckii* subsp. *lactis* ACA-DC 178, the CEP of *L. delbrueckii* subsp. *lactis* CRL 581 was able to hydrolyze the β-casein protein upstream of residue 144 (43). In contrast, more than 100 different peptides were identified in the hydrolysate of β-casein degraded by the proteinase of *L. lactis* (20). Four out of the 32 peptides described in the present study were also observed in the β-casein hydrolysate of *L. lactis*. These peptides were located at the C-terminal part of β-casein and included the Leu_{191}-Leu_{192}, Leu_{192}-Tyr_{193}, Tyr_{193}-Gln_{194}, and Leu_{198}-Gly_{199} bonds. Moreover, the C-terminal end of β-casein, which contains the bitter peptide f193-209, is highly susceptible to breakdown by CEP of *L. delbrueckii* subsp. *lactis* CRL 581 at the Tyr_{193}-Gln_{194}, Gln_{194}-Glu_{195}, Pro_{196}-Val_{197}, Val_{197}-Leu_{198}, Leu_{198}-Gly_{199}, Pro_{203}-Phe_{204}, and Pro_{205}-Ile_{206} bonds, as well as by the other previously described proteinases of LAB (17, 20, 23, 38, 40, 44, 45). In addition, the tripeptide Ile-Pro-Pro (f74-76) was identified in the β-casein hydrolysate generated by the CEP of *L. delbrueckii* subsp. *lactis* CRL 581 (Table 4 and Fig. 4B). This peptide inhibits angiotensin I-converting enzyme and has an antihypertensive effect (7, 12, 13, 19).

Thermophilic lactobacilli, including *L. delbrueckii* subsp. *lactis*, are involved in the production of hard cheeses, such as Italian- and Swiss-type cheeses. Nowadays, there is an increasing interest in the development of dairy health products. In this study, the pattern of α_{s1}- and β-casein breakdown by CEP of *L. delbrueckii* subsp. *lactis* CRL 581 is described. In particular, two new cleavage sites on the α_{s1}-casein (f1-23) fragment (Glu_{14}-Val_{15} and Glu_{18}-Asn_{19}), which have not been previously described for other CEPs of LAB (see Fig. 5), were identified for the CEP of *L. delbrueckii* subsp. *lactis* CRL 581. These data will contribute to the available knowledge on lactobacillus CEPs, which is limited compared to the best-described lactococcus CEP enzymes. Furthermore, since *L. delbrueckii* subsp. *lactis* CRL 581 releases the antihypertensive peptide Ile-Pro-Pro, this microorganism could be used as a functional cheese starter for its technological and health properties.

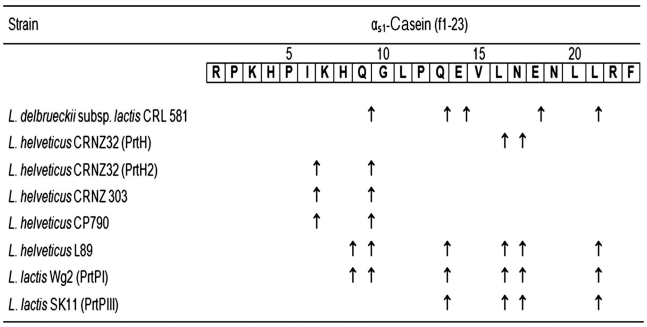

FIG. 5. Specificity of CEPs of LAB, including CEP from *L. delbrueckii* subsp. *lactis* CRL 581, toward the α_{s1}-casein fragment comprising residues 1 to 23. The cleavage sites are indicated by arrows.

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