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The Proteolytic System of *Lactobacillus sanfrancisco* CB1: Purification and Characterization of a Proteinase, a Dipeptidase, and an Aminopeptidase

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A cell envelope 57-kDa proteinase, a cytoplasmic 65-kDa dipeptidase, and a 75-kDa aminopeptidase were purified from *Lactobacillus sanfrancisco* CB1 sourdough lactic acid bacterium by sequential fast protein liquid chromatography steps. All of the enzymes are monomers. The proteinase was most active at pH 7.0 and 40°C, while aminopeptidase and dipeptidase had optima at pH 7.5 and 30 to 35°C. Relatively high activities were observed at the pH and temperature of the sourdough fermentation. The proteinase is a serine enzyme. Urea-polyacrylamide gel electrophoresis of digests of αs_1- and β-caseins showed differences in the pattern of peptides released by the purified proteinase and those produced by crude preparations of the cell envelope proteinases of *Lactobacillus delbrueckii* subsp. *bulgaricus* B397 and *Lactococcus lactis* subsp. *lactis* SK11. Reversed-phase fast protein liquid chromatography of gliadin digests showed a more-complex peptide pattern produced by the proteinase of *Lactobacillus sanfrancisco* CB1. The dipeptidase is a metalloenzyme with high affinity for dipeptides containing hydrophobic amino acids but had no activity on tripeptides or larger peptides. The aminopeptidase was also inhibited by metal-chelating agents and showed a broad N-terminal hydrolytic activity including di- and tripeptides. K_m values of 0.70 and 0.44 mM were determined for the dipeptidase on Leu-Leu and the aminopeptidase on Leu-*p*-nitroanilide, respectively.

Nitrogen is a growth-limiting factor for lactic acid bacteria in several food ecosystems (28). By a complex proteolytic system, lactic acid bacteria are able to degrade proteins into small peptides and amino acids, which can then be transported through the cell membrane (33). Proteolytic enzymes not only are indispensable for the nitrogen metabolism of lactic acid bacteria but also play a key role in food productions such as cheese ripening. As a consequence, several proteinases and peptidases have been isolated and characterized from lactic acid bacteria used in cheese making. The following have been studied: proteinase types P_I and P_{III} from the cell envelope of *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris* strains and other proteinases from *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus helveticus*, and *Lactobacillus delbrueckii* subsp. *lactis*; metal-dependent dipeptidases from *Lactococcus lactis* subsp. *cremoris*, *Streptococcus thermophilus*, and *Lactobacillus delbrueckii* subsp. *bulgaricus*; and general or specific aminopeptidases (type A, N, or C) with different substrate specificities and biochemical properties (26, 40, 43).

Lactic acid bacteria also populate food ecosystems other than dairy products. Lactic acid bacteria have been isolated or used as starters in sourdough wheat breads (35). *Lactobacillus sanfrancisco* has been found to be the dominant lactic acid bacterium in American, Swedish, German, Swiss, and Italian sourdoughs (19, 34–36, 39). The importance of *Lactobacillus sanfrancisco* has been related to its heterofermentative metabolism, short lag phase during dough acidification (17), production of volatile compounds (21), and symbiotic relationship with *Saccharomyces exiguus* (39). We previously studied the interaction between sourdough lactic acid bacteria and yeasts on the basis of the metabolism of amino acids (18) and the evolution of amino acids during sourdough fermentation and baking, and we defined *Lactobacillus sanfrancisco* CB1 to be the most proteolytic bacterium of the sourdough lactic acid bacteria (22). A general characterization and subcellular localization of the proteolytic enzymes in sourdough lactic acid bacteria have shown that *Lactobacillus sanfrancisco* CB1 contains particularly active proteinase, dipeptidase, and aminopeptidase enzymes (23). Amino acids and small peptides are important for rapid growth and acidification during sourdough fermentation and, as precursors, for the flavor development of leavened baked products (35).

The characterization and purification of proteolytic enzymes from nondairy lactic acid bacteria could extend the knowledge on the significance of this bacterial group in various food ecosystems and give a comparison between the biochemical properties of enzymes active on different protein sources.

In this article, we report the purification and characterization of a cell envelope proteinase and of an intracellular dipeptidase and an aminopeptidase from *Lactobacillus sanfrancisco* CB1 isolated from sourdoughs.

MATERIALS AND METHODS

Materials. Dipeptides, tripeptides, methionine enkephalin, *p*-nitroanilide (*p*NA) substrates, inhibitors, and gliadin were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Caseins (αs_1 and β) were prepared by isoelectric precipitation from bulk bovine skim milk and subsequent ion-exchange chromatography on DEAE-cellulose (DE-52; Whatman, Balston, England) with 10 mM imidazole buffer (pH 7.0) containing 4.5 M urea and 0.1% (vol/vol) 2-mercaptoethanol.

Prepacked fast protein liquid chromatography (FPLC) columns of Superose 12 HR 10/30 (30 by 1 cm inside diameter), phenyl-Superose HR 5/5 (5 by 0.5 cm inside diameter), Mono Q HR 5/5, PepRPC HR 5/5, and protein molecular mass standards, including phosphorylase *b* (94.0 kDa), bovine serum albumin (67.0 kDa), ovalbumin (43.0 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa), and blue dextran, were obtained from Pharmacia-LKB, Uppsala, Sweden.

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Filters of 0.22-μm pore size were obtained from Syrfil Filter, Nucleopore, Costar Corporation, Cambridge, Mass.

Microorganism, growth conditions, and subcellular fractionation. *Lactobacillus sanfrancisco* CB1 (syn. *Lactobacillus brevis* subsp. *lindneri*), isolated originally from Italian sourdoughs (19), was precultivated and cultivated (20 liters) in SDB broth (27) at 30°C. The 16-h cells were harvested at 10,000 × *g* for 10 min at 4°C and used for subcellular fractionation by lysozyme treatment in 50 mM Tris HCl (pH 7.5) buffer containing 24% (wt/vol) sucrose as described by Crow et al. (9). The only modification was that spheroplasts resuspended in isotonic buffer were sonicated for 40 s at 16 A/S (Sony Prep model 150; Sanyo, Tokyo, Japan) (23). The loosely associated cell surface and cytoplasmic fractions were used to purify the proteinase and the dipeptidase and aminopeptidase enzymes, respectively. No proteinase activity was detected in the supernatant fraction obtained from the cultural SDB broth (9).

Cellular fractions were freeze-dried (MOD E1PTB; Edwards, Milan, Italy), concentrated 10- to 20-fold by resuspending in 20 mM phosphate buffer (pH 6.5), and dialyzed for 24 h at 4°C against the same buffer.

Crude preparations of proteinases from the cell envelope of *Lactococcus lactis* subsp. *cremoris* SK11 and *Lactobacillus delbrueckii* subsp. *bulgaricus* B397 were obtained by the same procedure (9) and kindly supplied by the Department of Food Science, Agriculture Faculty of Norway, Ås.

Fructose-1,6-biphosphate aldolase (10) was used as a cytoplasmic marker. No aldolase activity was detected in the proteinase preparations, and more than 95% of this activity was located in the cytoplasm fraction.

Enzyme assays. Caseinolytic activity of the proteinase from *Lactobacillus sanfrancisco* CB1 was measured routinely in 10 mM phosphate buffer (pH 7.0) by the method of Twinning (41) with fluorescent casein (1.0% [wt/vol]) as the substrate. After 6 h of incubation at 30°C, the activity, expressed as an increase in arbitrary fluorescence units, was measured with a fluorescence high-performance liquid chromatography (HPLC) monitor (Schimadzu Corporation, Kyoto, Japan). Activity in chromatographic αs_1 and β fractions (1 mg/ml final concentration) and gliadin (dissolved in 70% [vol/vol]) ethanol and used at a final concentration of 4 mg/ml) was measured after incubation at 30°C for 14 h. The incubation mixture contained 0.02% (wt/vol) NaN_3.

The specific activities of the cell envelope proteinases from *Lactococcus lactis* subsp. *cremoris* SK11 and *Lactobacillus delbrueckii* subsp. *bulgaricus* B397 on fluorescent casein were about 235 and 178 U/mg of protein, respectively. As a consequence, the purified proteinase solution from *Lactobacillus sanfrancisco* CB1 and these preparations were diluted proportionally to have the same activity on fluorescent casein (pH 7.0 and 30°C) and used in these concentrations in the various assays.

Dipeptidase activity on Leu-Leu substrate and the hydrolysis of tripeptides and methionine enkephalin were determined by the Cd-ninhydrin method of Doi et al. (11). Two hundred microliters of 10 mM phosphate buffer (pH 7.0) was mixed and incubated with the enzyme solution (50 μl) and Leu-Leu (20 mM; 25 μl) at 35°C for 45 min. The reaction was terminated by the addition of 750 μl of Cd-ninhydrin reagent. After incubation at 85°C for 5 min, the solution was cooled, and the A_{505} was measured. A unit of dipeptidase activity was defined as the amount of enzyme that hydrolyzed 1 nmol of Leu-Leu per min.

Aminopeptidase activity was measured on Leu-*p*NA by the method of El Soda and Desmazeaud (13). The microassay mixture contained 200 μl of 10 mM phosphate buffer (pH 7.0), 25 μl of Leu-*p*NA (20 mM in methanol), and 50 μl of enzyme solution. After incubation for 30 min at 35°C, the reaction was stopped with 10% (vol/vol) acetic acid, and the activity was determined by reading the A_{410}. A unit of aminopeptidase activity was defined as the amount of enzyme that produced an increase in A_{410} of 0.01 per min. The specific activities of all of the enzymes were defined as the units of enzyme activity per milligram of protein used in the assays.

Electrophoresis. Urea-polyacrylamide gel electrophoresis (urea-PAGE) was used to separate peptides released from αs_1 and β caseins by the proteinases. Electrophoresis was performed by the method of Andrews (1); gels were stained as described by Blakesley and Boezi (4).

Sodium dodecyl sulfate (SDS)-PAGE was conducted by the Laemmli procedure (29); gels contained 12% acrylamide (separation distance, 10 cm; gel thickness, 1 mm).

RP-FPLC. Reversed-phase (RP)-FPLC in a PepRPC HR 5/5 column was used to separate 1% trifluoroacetic acid (TFA)-soluble peptides released from αs_1 casein and gliadin by the purified proteinases. Chromatographic conditions were as described by Stepaniak and Fox (38).

Protein determination. Protein was determined by the method of Bradford (7) with bovine serum albumin as the standard.

Isolation and purification of the proteolytic enzymes. (i) Chromatography on Q-Sepharose. The loosely associated cell surface or the cytoplasmic fraction, concentrated by freeze-drying, resuspended in 20 mM phosphate buffer (pH 6.5), and filtered through a 0.22-μm-pore-size filter, was separately applied to a Q-Sepharose HR 16/50 column (50 by 1.6 cm inside diameter) previously equilibrated with the same buffer. After loading, proteins were eluted with a linear NaCl gradient, 0 to 0.6 M, at a flow rate of 18 ml/h. The fractions with the highest proteinase, dipeptidase, or aminopeptidase activity were pooled, dialyzed for 24 h at 4°C against distilled water, and concentrated 10-fold by freeze-drying.

(ii) Gel filtration on Sephacryl 200 or chromatography on hydroxyapatite. Fractions containing proteinase or aminopeptidase activity from the previous step were redissolved in a small volume of 50 mM phosphate buffer (pH 6.5) and further purified by gel filtration on Sephacryl 200 (column size, 60 by 2.0 cm inside diameter), equilibrated with 50 mM phosphate buffer (pH 6.5) containing 0.1 M NaCl. Elution with the same buffer was at a flow rate of 11 ml/h. The most active fractions were pooled.

By use of the protocol for purifying a dipeptidase from *Pseudomonas fluorescens* ATCC 948 (20), the fractions containing dipeptidase activity were applied to a hydroxyapatite HR 16/10 column that had been equilibrated with 10 mM phosphate buffer (pH 7.0) containing 10 mM NaCl. The enzyme was eluted (flow rate, 6 ml/h) with a linear gradient of NaCl from 0.01 to 0.2 M, and the most active fractions were pooled.

(iii) Chromatography on phenyl-Superose or gel filtration on Superose 12. After dialysis (24 h at 4°C against distilled water) and freeze-drying, the 10-fold-concentrated fractions with proteinase or aminopeptidase activity were further purified on an FPLC phenyl-Superose HR 5/5 column that had been equilibrated with 50 mM phosphate buffer (pH 7.0) containing 1.7 M $(NH_4)_2SO_4$. Both of the enzyme fractions were resuspended in 10 mM phosphate buffer (pH 7.0) containing 1.7 M $(NH_4)_2SO_4$, separately applied to the column, and eluted with a reversed linear gradient of 1.7 to 0 M $(NH_4)_2SO_4$ at a flow rate of 0.4 ml/min. The fractions which showed the highest proteinase or aminopeptidase activity were desalted by dialysis against distilled water (24 h at 4°C) and concentrated 10-fold by freeze-drying.

Pooled fractions with dipeptidase activity from the hydroxyapatite column were loaded onto an FPLC Superose 12 HR 10/30 column. The column was eluted with 50 mM phosphate buffer containing 0.15 M NaCl (pH 7.0) at a flow rate of 0.4 ml/min. Fractions with Leu-Leu hydrolyzing activity were pooled, dialyzed, and concentrated.

(iv) Chromatography on Mono Q or phenyl-Superose. Aminopeptidase from *Lactobacillus sanfrancisco* CB1 was finally purified on an FPLC Mono Q HR 5/5 column. After loading of the sample, elution was performed with a linear gradient of 0 to 0.3 M NaCl at a flow rate of 0.75 ml/min with 20 mM bis-Tris-propane (pH 6.5). One-milliliter fractions were collected, and the two fractions corresponding to the peak of aminopeptidase activity were pooled, dialyzed (24 h at 4°C against distilled water), freeze-dried, and used for characterization. The same chromatography step was used for the fractions containing proteinase activity.

Dipeptidase from *Lactobacillus sanfrancisco* CB1 was finally purified on an FPLC phenyl-Superose column under the conditions described for the purification of proteinase and aminopeptidase.

(v) Second chromatography on Mono Q. The purification to homogeneity of the proteinase from *Lactobacillus sanfrancisco* CB1 required a further elution with a Mono Q column, which was performed as described previously.

Characterization of the proteolytic enzymes. (i) molecular mass measurement. The relative molecular masses of the purified proteinase, dipeptidase, and aminopeptidase were estimated by FPLC gel filtration and SDS-PAGE. Gel filtration (mean of four determinations) was conducted on a Superose 12 HR 10/30 column calibrated with protein molecular mass standards. Equilibrium and elution (0.4 ml/min) were performed with 50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl.

(ii) pH and temperature optima. The effect of pH on the proteinase activity was examined in the pH range of 5 to 11, by use of a universal buffer composed of boric acid (57 mM), citric acid (33 mM), NaH_2PO_4 (33 mM), NaOH (1 M), and various amounts of 0.1 M HCl. A wide range of pH values (4.0 to 11.0) was considered for the dipeptidase and aminopeptidase. The temperature dependence was determined in 10 mM phosphate buffer (pH 7.0) in the range of 5 to 55°C. Fluorescent casein, Leu-Leu, and Leu-*p*NA were used as substrates for proteinase, dipeptidase, and aminopeptidase following the enzyme assays described previously.

(iii) Enzyme kinetics. Enzyme solutions of dipeptidase and aminopeptidase were incubated with various concentrations of Leu-Leu and Leu-*p*NA, with a final substrate concentration ranging from 0.11 to 2.6 mM. The plot representation of Hanes (24) was used to calculate the Michaelis-Menten constant (K_m).

(iv) Effect of chemical reagents and divalent cations. A mixture containing the purified enzyme solutions and 1.0 mM (final concentration) inhibitors or divalent cations in 50 mM phosphate buffer was incubated for 30 min at 30°C. Reactions were initiated by adding the specific substrates, and enzyme activities were assayed under standard conditions. Controls to eliminate the interference of dithiothreitol, β-mercaptoethanol, and cysteine in the Cd-ninhydrin method were considered.

RESULTS

Purification of the proteolytic enzymes. The yields and degrees of purification of the proteolytic enzymes are summarized in Table 1. The proteinase from the cell envelope of *Lactobacillus sanfrancisco* CB1 was purified 62-fold with a recovery of 14% of the enzyme activity. Chromatography on phenyl-Superose gave the highest partial increase of specific activity, and repeated elution on Mono Q was indispensable for obtaining an electrophoretically pure enzyme. Traces of

TABLE 1. Purification of the proteinase, dipeptidase, and aminopeptidase from *Lactobacillus sanfrancisco* CB1

Enzyme and purification step	Amt of protein (mg)	Total activity (U)	Sp act (U/mg)	Purification (fold)	Activity yield (%)
Proteinase					
Cell-free supernatant	316.7	2,016.6	6.37	1	100
Q-Sepharose	48.5	945.6	19.50	3.0	47
Sephacryl 200	21.2	612.7	28.90	4.5	30
Phenyl-Superose	4.9	542.8	110.77	17.4	27
Mono Q	1.7	315.7	185.70	29.2	16
Mono Q	0.7	274.2	391.71	61.5	14
Dipeptidase					
Cell extract	995.5	815.3	0.81	1	100
Q-Sepharose	315.2	598.2	1.90	2.3	73
Hydroxyapatite	36.7	513.5	13.99	17.3	63
Superose 12	3.2	311.5	97.34	120.2	38
Phenyl-Superose	0.9	158.2	175.77	217.0	19
Aminopeptidase					
Cell extract	995.5	927.6	0.93	1	100
Q-Sepharose	207.9	662.5	3.19	3.4	71
Sephacryl 200	112.3	582.1	5.18	5.6	63
Phenyl-Superose	11.2	376.7	33.63	36.2	41
Mono Q	1.4	171.8	122.71	132.0	18

caseinolytic activity, probably due to proteinase autolysis, were removed by chromatography on Sephacryl 200 and phenyl-Superose.

Chromatography of the cytoplasm from *Lactobacillus sanfrancisco* CB1 on Q-Sepharose resolved two major peaks with dipeptidase activity and one peak which corresponded to the aminopeptidase. Only the peak containing the highest specific dipeptidase activity was considered further. After chromatography on hydroxyapatite, the gel filtration on Superose 12 removed substantial amounts of nondipeptidase proteins. The dipeptidase was purified 217-fold with a final yield of 19% and a specific activity of 176 U/mg. By using the same protocol adopted for the proteinase purification, except for the fifth step on Mono Q, the purified aminopeptidase was enriched 132-fold with a recovery of 18% of the enzyme activity.

SDS-PAGE showed that the enzyme preparations were homogeneous (Fig. 1).

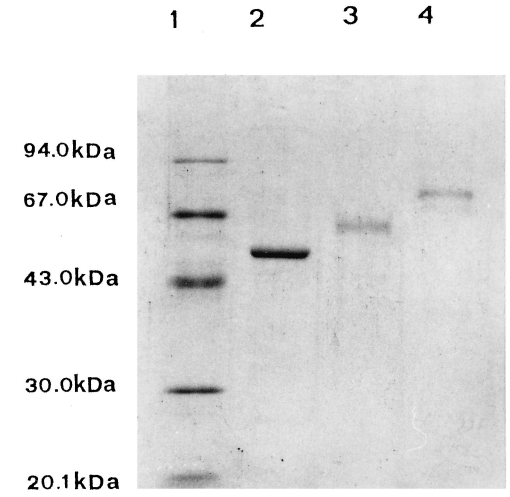

FIG. 1. SDS-PAGE of the purified enzymes from *Lactobacillus sanfrancisco* CB1. Lanes: 1, reference proteins (see Materials and Methods); 2, proteinase; 3, dipeptidase; 4, aminopeptidase.

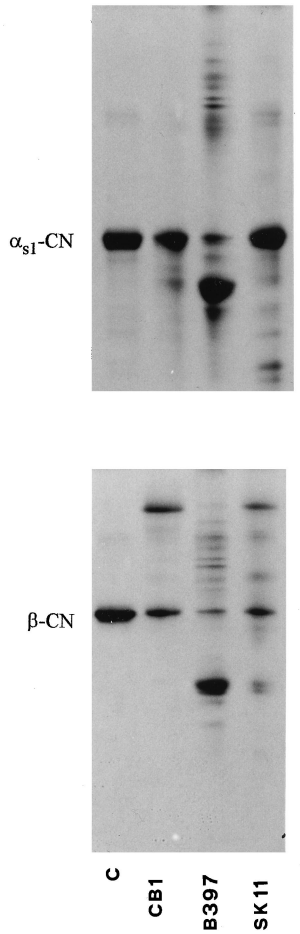

FIG. 2. Urea-PAGE electropherograms of αs_1 and β caseins (αs_1-CN and β-CN) incubated with the proteinases from *Lactobacillus sanfrancisco* CB1, *Lactobacillus delbrueckii* subsp. *bulgaricus* B397, and *Lactococcus lactis* subsp. *cremoris* SK11. C, control incubated for 14 h without proteinases.

Characterization of the proteolytic enzymes. (i) Molecular mass. Molecular masses (±1.5 kDa) of 57, 65, and 75 kDa were estimated by gel filtration on Superose 12 for the proteinase, dipeptidase, and aminopeptidase from *Lactobacillus sanfrancisco* CB1, respectively (data not shown). Determinations from SDS-PAGE compared well with these results, showing that these enzymes are monomers (Fig. 1).

(ii) pH and temperature optima. The optimal caseinolytic activity was found at pH 7.0, while dipeptidase and aminopeptidase exhibited the same optima at pH 7.5. All enzyme activities were higher than 30% of the pH 7.0 to 7.5 maximal values when the H^+ concentration was increased or decreased about 500-fold (pH 4.0 or 11.0) from that region.

The temperature optima for aminopeptidase, dipeptidase, and proteinase were 30, 35, and 40°C as determined by specific enzyme assays. Seventy to 90% of the maximum activity on fluorescent casein was maintained in the range of 30 to 35°C.

(iii) Substrate specificity. Urea-PAGE of β-casein hydrolysates showed differences in the peptide patterns produced by the proteinases from *Lactobacillus sanfrancisco* CB1, *Lactococcus lactis* subsp. *cremoris* SK11, and *Lactobacillus delbrueckii* subsp. *bulgaricus* B397 (Fig. 2). The proteinase from *Lactobacillus sanfrancisco* CB1 liberated one main peptide, which was also produced by the lactococcal proteinase. As shown in the

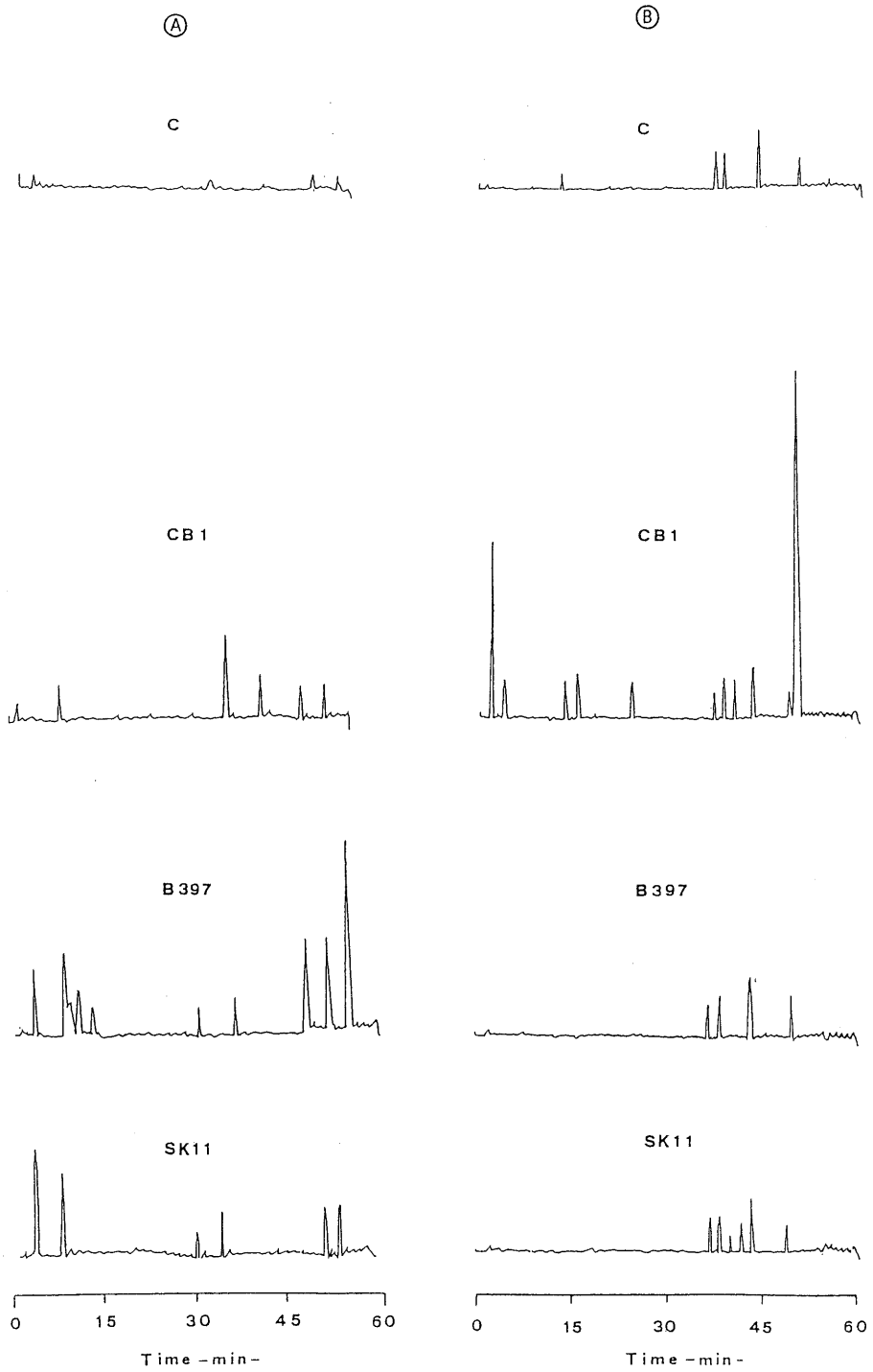

FIG. 3. RP-FPLC chromatograms of 1% TFA-soluble peptides released from αs_1 casein (A) and gliadin (B) by the proteinases from *Lactobacillus sanfrancisco* CB1, *Lactobacillus delbrueckii* subsp. *bulgaricus* B397, and *Lactococcus lactis* subsp. *cremoris* SK11. C, control incubated for 14 h without proteinases.

electropherograms, except when incubated with the proteinase of *Lactobacillus delbrueckii* subsp. *bulgaricus* B397, αs_1 casein was slightly hydrolyzed. RP-FPLC chromatograms of TFA-soluble peptides released from αs_1 casein confirmed either a more complex pattern of peptides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* B397 or differences in the specificity of the proteinase from *Lactobacillus sanfrancisco* CB1 (Fig. 3A). The same results were obtained with a *Lactobacillus sanfrancisco* CB1 strain, which, after curing, did not harbor the cryptic plasmid (ca. 17 kbp) contained in the wild type (data not shown). Lönner et al. (30) previously suggested that the proteolytic activity of *Lactobacillus sanfrancisco* strains isolated from sourdoughs could be coded by plasmids. RP-FPLC chromatograms of TFA-soluble peptides released from gliadin (Fig. 3B) showed a reduced activity of the proteinases from *Lactobacillus delbrueckii* subsp. *bulgaricus* B397 and *Lactococ-*

TABLE 2. Substrate specificity of the dipeptidase and aminopeptidase from *Lactobacillus sanfrancisco* CB1

Substrate for dipeptidase	% Dipeptidase activity	Substrate for aminopeptidase	% Aminopeptidase activity
Leu-Leu	100	Leu-*p*NA	100
D-Leu-Leu	56	Pro-*p*NA	0
Leu-D-Leu	0	Lys-*p*NA	98
Pro-Gly	0	Ala-*p*NA	64
Val-Leu	77	Phe-*p*NA	77
Leu-Pro	0	Arg-*p*NA	96
Ala-Phe	30	His-*p*NA	30
Leu-Gly	73	Iso-*p*NA	103
His-Leu	58	Trp-*p*NA	58
Lys-Lys	86	Met-*p*NA	86
Lys-Ala	93	Val-*p*NA	7
Ala-Pro	0	Glu-*p*NA	3
Phe-Val-*p*NA	0	Asp-*p*NA	4
Gly-Pro-*p*NA	0	Gly-Pro-*p*NA	0
Leu-Leu-Leu	0	Gly-Phe-*p*NA	0
Leu-Gly-Gly	0	Leu-Leu	100
Ala-Ala-Ala	0	Ser-Tyr	62
Leu-*p*NA	0	Leu-Gly	88
Lys-*p*NA	0	Phe-Val	12
Met-enkephalin	0	Tyr-Pro	0
		Leu-Leu-Leu	72
		Leu-Gly-Gly	65
		Phe-Gly-Gly	51
		Met-enkephalin	0

cus lactis subsp. *cremoris* SK11 and a complex peptide pattern produced by the *Lactobacillus sanfrancisco* CB1 enzyme. Two main peptides which eluted at the beginning and in the last part of the acetonitrile gradient characterized the activity of the CB1 proteinase on gliadin.

The hydrolytic action of dipeptidase and aminopeptidase towards various peptides and *p*NA substrates is summarized in Table 2. Of the dipeptides tested, substrates with an N-terminal hydrophobic amino acid were cleaved at the highest rate, which indicated that the N-terminal amino acid of a peptide plays an important role in enzyme specificity. Peptides containing a D-isomer at the N terminus were also hydrolyzed quite rapidly, as were dipeptides of type Lys-X. Tripeptides, larger peptides (methionine enkephalin), and dipeptides containing a proline residue and Leu-D-Leu (D-isomer at the C-terminal of the dipeptide) were not hydrolyzed. The purified enzyme was unable to cleave amino acid nitroanilides such as Leu-*p*NA and Lys-*p*NA.

A broad N-terminal hydrolytic ability was demonstrated by the purified aminopeptidase from *Lactobacillus sanfrancisco* CB1. The enzyme had high affinity for positively charged amino acids such as Lys and Arg or for hydrophobic amino acids such as Leu and Iso but had no activity toward Glu and Pro-*p*NA (Table 2). A range of di- and tripeptides were also cleaved by the aminopeptidase. Hydrolysis of longer peptides (methionine enkephalin), dipeptidyl derivatives such as Gly-Pro-*p*NA and Gly-Phe-*p*NA, and dipeptides involving Pro residues could not be detected.

(iv) Enzyme kinetics. K_m values of 0.70 mM on Leu-Leu and 0.44 mM on Leu-*p*NA were determined for the dipeptidase and the aminopeptidase, respectively. These values compared well with those determined for the same enzymes in different lactic acid bacterial strains (2, 3, 42).

(v) Effect of chemical reagents and divalent cations. The effects of activators or inhibitors on the proteolytic enzymes of *Lactobacillus sanfrancisco* CB1 are summarized in Table 3. Sulfydryl-reducing agents or metal chelators had no effect on the activity of the proteinase. *p*-Chloromercuribenzoate and iodoacetic acid caused moderate inhibition, while the serine proteinase inhibitor phenylmethylsulfonyl fluoride caused extensive inhibition at 1.0 mM. Mg^{2+}, Ca^{2+}, and especially Co^{2+} caused an increase in activity, while the remaining metals caused a reduction.

The dipeptidase and aminopeptidase were strongly inhibited by the metal-chelating agents EDTA and 1,10-phenanthroline. While the aminopeptidase was partially inhibited by *p*-chloromercuribenzoate and slightly activated by dithiothreitol, β-mercaptoethanol and cysteine, the same compounds that can reduce thiol groups in the enzyme, considerably inhibited the dipeptidase. Mg^{2+}, Ca^{2+}, and Co^{2+} activated the dipeptidase and Cu^{2+}, Ni^{2+}, Fe^{2+}, and Hg^{2+} almost completely abolished both activities.

DISCUSSION

Lactobacillus sanfrancisco CB1, a key sourdough lactic acid bacterium, has been shown to have a particular capacity to degrade proteins or peptides during sourdough fermentation (22). A cell envelope-associated serine proteinase, a metal-dependent dipeptidase, and a general aminopeptidase are the main enzymes which characterize the proteolytic system of *Lactobacillus sanfrancisco* CB1 (23).

Compared with studies on *Lactococcus* strains (40), few studies have been done on proteinases from *Lactobacillus* spp. To our knowledge, after some characterization of the cell envelope proteinases from *Lactobacillus helveticus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Lactobacillus delbrueckii* subsp. *lactis*, and *Lactobacillus casei* subsp. *casei* (14, 47), only proteinases from *Lactobacillus helveticus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* have been purified (15, 31, 46). Contrary to Ezzat et al. (15), who after performing an initial chromatography on DEAE-cellulose observed three peaks of caseinolytic activity in the cell wall fraction of *Lactobacillus delbrueckii* subsp. *bulgaricus*, we found that only one proteinase is located in the cell envelope of *Lactobacillus sanfrancisco*

TABLE 3. Effect of chemical reagents and divalent cations on the activity of the proteinase, dipeptidase, and aminopeptidase from *Lactobacillus sanfrancisco* CB1

Reagent or cation[a]	Activity (%)		
	Proteinase	Dipeptidase	Aminopeptidase
DTT	88	32	115
β-Mercaptoethanol	92	26	107
Cysteine	85	54	111
NEM	83	97	99
PCMB	68	92	45
PMSF	15	70	94
EDTA	85	4	7
1,10-Phenanthroline	82	12	16
Iodoacetic acid	65	95	73
Mg^{2+}	112	130	92
Ca^{2+}	108	132	95
Cu^{2+}	25	10	2
Mn^{2+}	68	54	69
Co^{2+}	115	127	35
Ni^{2+}	10	7	3
Zn^{2+}	45	10	2
Fe^{2+}	37	28	3
Hg^{2+}	12	3	4

[a] DTT, dithiothreitol; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethylsulfonyl fluoride.

CB1. Like the proteinase from this study, the purified proteinase from *Lactobacillus helveticus* CP790 is a serine enzyme with a similar molecular mass, 45 kDa, and pH and temperature optima of 6.5 to 7.0 and 40°C, respectively (46). A metal-dependent proteinase insensitive to phenylmethylsulfonyl fluoride has been characterized for *Lactobacillus delbrueckii* subsp. *bulgaricus* (15), while other *Lactobacillus helveticus* strains exhibited proteinases with different molecular masses (ca. 180 kDa). As shown by the electropherograms and the RP-FPLC chromatograms of αs_1 and β caseins, the P_{III} proteinase from *Lactococcus lactis* subsp. *cremoris* SK11 and the cell envelope proteinase from *Lactobacillus delbrueckii* subsp. *bulgaricus* B397 both differed from the enzyme of *Lactobacillus sanfrancisco* CB1. On the contrary, similarity among *Lactobacillus helveticus* L89 (31) and *Lactobacillus casei* subsp. *casei* IFPL 731 (16) proteinases and P_I and P_{III} lactococcal proteinases has been shown. All of the proteinases in this study showed higher activity on β casein than on αs_1 casein, but the *Lactobacillus sanfrancisco* CB1 proteinase differed as a result of the limited hydrolysis of caseins and the considerable capacity to produce TFA-soluble peptides from gliadin. Gliadin is the major component of the gluten which is formed during dough mixing (37). During sourdough fermentation, the lactic acid bacteria isolated from sourdoughs have also been shown to possess higher acidification rates (17) and to produce more volatile compounds (21) than reference strains from different sources.

To date, dipeptidases in cellular extracts of dairy *Lactobacillus* species have been studied (13), and only a dipeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* (43) has been purified and thoroughly characterized. The dipeptidase from *Lactobacillus sanfrancisco* CB1 is a monomer with a molecular mass of about 65 kDa, which compared well with the values for the monomer dipeptidases isolated from *Lactococcus lactis* subsp. *cremoris* Wg2 (42) and *Lactobacillus delbrueckii* subsp. *bulgaricus* B14 (43), but differs from the dipeptidase of *Lactococcus lactis* subsp. *cremoris* H61 (25), which had a molecular mass of about 100 kDa and functioned as a dimer. Common features within lactic acid bacterial dipeptidases are an optimum pH in the range of 7.0 to 8.0, inhibition by metal chelators and to a lesser extent by reagents which block sulfhydryl groups, requirements for metal ions (Co^{2+}), and no activity on tripeptides, pentapeptides, and amino acid-nitroanilides. In particular, the higher activity on Leu-Leu rather than on Leu-Gly and a preferential hydrolysis of dipeptides containing hydrophobic amino acids (Leu, Ala, or Phe) showed a considerable similarity between the substrate specificity of the dipeptidase from this study and that isolated from *Lactobacillus delbrueckii* subsp. *bulgaricus* B14 (43). After glutamic acid and proline, the hydrophobic amino acids are the major amino acid residues contained in the gluten of wheat flour (8). Considerable iminopeptidase activity toward Pro-X peptides has also been shown in the crude cytoplasm of *Lactobacillus sanfrancisco* CB1 (23). With respect to that found for *Lactobacillus sanfrancisco* CB1 dipeptidase (35°C), a higher optimum temperature (45 to 50°C) has been reported for dipeptidases from other lactic acid bacteria (25, 42).

The aminopeptidase of *Lactobacillus sanfrancisco* CB1 has several features in common with other aminopeptidases purified from different *Lactobacillus* species. Metal-dependent enzymes having a molecular mass in the range of 70 to 100 kDa, with Lys-*p*NA or Leu-*p*NA as the preferential substrate, and with a pH optimum at 7.0 to 7.5 have also been reported in *Lactobacillus helveticus* ITGL1 (5), *Lactobacillus casei* subsp. *rhamnosus* (3), *Lactobacillus casei* subsp. *casei* (2), *Lactobacillus delbrueckii* subsp. *lactis* (12), and *Lactobacillus delbrueckii* subsp. *bulgaricus* (6, 44, 45). Therefore, as proposed by the classification of Tan et al. (40), the enzyme of *Lactobacillus sanfrancisco* CB1 is an aminopeptidase type N. On the contrary, high-molecular-weight dimers or trimers, inactivated by thiol-group inhibitors, have been reported in *Lactobacillus delbrueckii* subsp. *bulgaricus* (44, 45), which showed a very complex peptidase system. The low optimum of activity at 30°C distinguished the aminopeptidase of *Lactobacillus sanfrancisco* CB1 from the other enzymes of lactic acid bacteria. The high activity on *p*NA derivatives and on dipeptides and tripeptides which have hydrophobic amino acid or diaminomonocarboxylic acid (Lys or Arg) at the N terminus and the sensitivity to *p*-chloromercuribenzoate are features in common for the aminopeptidase of this study and the enzymes purified from *Lactobacillus helveticus* LHE-511 (32) and *Lactobacillus casei* subsp. *rhamnosus* S93 (3).

This is the first report on the purification to homogeneity and characterization of the proteolytic enzymes from *Lactobacillus sanfrancisco* CB1. We showed several similarities between the proteinase, dipeptidase, and aminopeptidase from this microorganism and those from lactic acid bacteria from dairy products studied previously. Particular features were also pointed out: (i) the specific capacity of the proteinase of *Lactobacillus sanfrancisco* CB1 to hydrolyze gliadin rather than casein fractions; (ii) the high affinity of the dipeptidase and aminopeptidase of *Lactobacillus sanfrancisco* CB1 for peptides containing hydrophobic amino acids; (iii) the low temperature optima of these exopeptidase activities; and (iv) the relatively high activity of the proteinase at the pH (4.5 to 5.5) and temperature (30 to 35°C) of the sourdough fermentation.

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REFERENCES

1. **Andrews, A. T.** 1983. Proteinases in normal bovine milk and their action on caseins. J. Dairy Res. **50:**45–55.
2. **Arora, G., and B. H. Lee.** 1992. Purification and characterization of an aminopeptidase from *Lactobacillus casei* subsp. *casei*. J. Dairy Sci. **75:**700–712.
3. **Arora, G., and B. H. Lee.** 1994. Purification and characterization of an aminopeptidase from *Lactobacillus casei* subsp. *rhamnosus*. Biotechnol. Appl. Biochem. **19:**179–192.
4. **Blakesley, R. W., and J. A. Boezi.** 1977. A new staining technique for proteins in polyacrylamide gels using Coomassie brilliant blue G250. Anal. Biochem. **82:**580–582.
5. **Blanc, B., P. Laloi, D. Atlan, C. Gilbert, and R. Portalier.** 1993. Two cell-wall-associated aminopeptidases from *Lactobacillus helveticus* and the purification and characterization of APII from strain ITGL1. J. Gen. Microbiol. **139:**1441–1448.
6. **Bockelmann, W., Y. Schulz, and M. Teuber.** 1992. Purification and characterization of an aminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. Int. Dairy J. **2:**95–107.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
8. **Carnovale, E., and F. Miuccio.** 1989. Contenuto in aminoacidi, p. 36. *In* Istituto Nazionale della Nutrizione (ed.), Tabelle di composizione degli alimenti. INN, Rome.
9. **Crow, V. L., R. Holland, and T. Coolbear.** 1993. Comparison of subcellular fractionation methods for *Lactobacillus lactis* subsp. *lactis* and *Lactobacillus lactis* subsp. *cremoris*. Int. Dairy J. **3:**599–611.
10. **Crow, V. L., and T. Thomas.** 1982. D-Tagatose 1,6-diphosphate aldolase from lactic streptococci: purification, properties and use in measuring intracellular tagatose 1,6-diphosphate. J. Bacteriol. **151:**600–608.
11. **Doi, E., D. Shibata, and T. Matoba.** 1981. Modified colorimetric ninhydrin methods for peptide assay. Anal. Biochem. **118:**173–182.
12. **Eggiman, B., and M. Bachmann.** 1980. Purification and partial characterization of an aminopeptidase from *Lactobacillus lactis*. Appl. Environ. Microbiol. **40:**876–882.
13. **El Soda, M., and M. Desmazeaud.** 1982. Les peptides des *Lactobacillus* du groupe thermobacterium. I. Mise en évidence de ces activitiés chez *Lacto-*

bacillus helveticus, *L. acidophilus*, *L. lactis* et *L. bulgaricus*. Can. J. Microbiol. **28:**1181–1187.
14. **Ezzat, N., M. El Soda, C. Bouillane, C. Zevaco, and P. Blanchard.** 1985. Cell wall associated proteinases in *Lactobacillus helveticus*, *Lactobacillus bulgaricus* and *Lactobacillus lactis*. Milchwissenschaft **40:**140–143.
15. **Ezzat, N., C. Zevaco, M. El Soda, and J. C. Gripon.** 1987. Partial purification and characterization of a cell wall associated proteinase from *Lactobacillus bulgaricus*. Milchwissenschaft **42:**95–97.
16. **Fernendez de Palencia, P., C. Pelaez, T. Requema, and M. Martin-Hernandez.** 1995. Release and partial characterization of cell-envelope proteinases from *Lactococcus lactis* subsp. *lactis* IFPL 359 and *Lactobacillus casei* subsp. *casei* IFPL 731 isolated from raw goat's-milk cheese. Z. Lebensm. Unters. Forsch. **201:**87–90.
17. **Gobbetti, M., A. Corsetti, and S. De Vincenzi.** 1995. The sourdough microflora. Characterization of heterofermentative lactic acid bacteria based on acidification kinetics and impedance test. Ital. J. Food Sci. **2:**103–113.
18. **Gobbetti, M., A. Corsetti, and J. Rossi.** 1994. The sourdough microflora. Interactions between lactic acid bacteria and yeasts: metabolism of amino acids. World J. Microbiol. Biotechnol. **10:**275–279.
19. **Gobbetti, M., A. Corsetti, J. Rossi, F. De Rosa, and S. De Vincenzi.** 1994. The sourdough microflora. Identification and clustering of lactic acid bacteria and yeasts from sourdoughs of Central Italy. Ital. J. Food Sci. **1:**85–94.
20. **Gobbetti, M., and P. F. Fox.** Purification and characterization of a dipeptidase from *Pseudomonas fluorescens* ATCC 948. J. Dairy Sci., in press.
21. **Gobbetti, M., M. S. Simonetti, A. Corsetti, F. Santinelli, J. Rossi, and P. Damiani. 1995.** Volatile compound and organic acid productions by mixed wheat sour dough starters: influence of fermentation parameters and dynamics during baking. Food Microbiol. **12:**497–507.
22. **Gobbetti, M., M. S. Simonetti, J. Rossi, L. Cossignani, A. Corsetti, and P. Damiani.** 1994. Free D- and L-amino acid evolution during sourdough fermentation and baking. J. Food Sci. **59:**881–884.
23. **Gobbetti, M., E. Smacchi, P. F. Fox, L. Stepaniak, and A. Corsetti.** The sourdough microflora. Cellular localization and characterization of proteolytic enzymes in lactic acid bacteria. Food Sci. Technol., in press.
24. **Hanes, C. S.** 1932. The determination of enzyme constants, p. 62. *In* M. Doxman and E. C. Webb (ed.), Enzymes. Longman Group Ltd., London, England.
25. **Hwang, I. K., S. Kaminogawa, and K. Yamauchi.** 1981. Purification and properties of a dipeptidase from *Streptococcus cremoris*. Agric. Biol. Chem. **45:**159–165.
26. **Khalid, N. M., and E. H. Marth.** 1990. Lactobacilli. Their enzymes and role in ripening and spoilage of cheese: a review. J. Dairy Sci. **73:**2669–2684.
27. **Kline, L., and T. F. Sugihara.** 1971. Microorganisms of San Francisco sourdough bread process. II. Isolation and characterization of undescribed bacterial species responsible for souring activity. Appl. Microbiol. **21:**459–465.
28. **Laan, H., E. J. Smid, P. S. T. Tan, and W. N. Konings.** 1989. Enzymes involved in the degradation and utilization of casein in *Lactococcus lactis*. Neth. Milk Dairy J. **43:**327–345.
29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
30. **Lönner, C., K. Preve-Akesson, and S. Ahrné.** 1990. Plasmid contents of lactic acid bacteria isolated from different types of sour doughs. Curr. Microbiol. **20:**201–207.
31. **Martin-Hernandez, M. C., A. C. Alting, and F. A. Exterkate.** 1994. Purification and characterization of the mature, membrane-associated cell-envelope proteinase of *Lactobacillus helveticus* L89. Appl. Microbiol. Biotechnol. **40:** 828–834.
32. **Miyakawa, H., S. Kobayashi, S. Shimamura, and M. Tomita.** 1992. Purification and characterization of an aminopeptidase from *Lactobacillus helveticus* LHE-511. J. Dairy Sci. **75:**27–35.
33. **Smid, E. J., B. Poolman, and W. N. Konings.** 1991. Casein utilisation by lactococci. Appl. Environ. Microbiol. **57:**2447–2452.
34. **Spicher, G.** 1983. Baked goods, p. 1–80. *In* H. J. Rehm. and G. Reed (ed.), Biotechnology, vol. 5. Verlag Chemie, Weinheim, Germany.
35. **Spicher, G.** 1987. The microflora of sourdough. XXII. Communication: the *Lactobacillus* species of wheat sourdough. Z. Lebensm. Unters. Forsch. **184:** 300–303.
36. **Spicher, G., and C. Lönner.** 1985. The microflora of sourdough. XXI. Communication: the *Lactobacillus* species from Swedish Bakeries. Z. Lebensm. Unters. Forsch. **181:**9–13.
37. **Stear, C. A.** 1990. Chemical bonding during doughmaking, p. 60–85. *In* Handbook of breadmaking technology. Elsevier Science Publishers Ltd., Essex, England.
38. **Stepaniak, L., and P. F. Fox.** 1995. Characterization of the principal intracellular endopeptidase from *Lactococcus lactis* subsp. *lactis*. Int. Dairy J. **5:**699–713.
39. **Sugihara, T. F., L. Kline, and L. B. McCredy.** 1970. Nature of the San Francisco sour dough French bread process. I. Microbiological aspects. Baker's Dig. **44:**50–52.
40. **Tan, P. S. T., B. Poolman, and W. N. Konings.** 1993. Proteolytic enzymes of *Lactococcus lactis*. J. Dairy Res. **60:**269–286.
41. **Twinning, S.** 1984. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. Anal. Biochem. **143:**30–34.
42. **van Boven, A., P. S. T. Tan, and W. N. Konings.** 1988. Purification and characterization of a dipeptidase from *Streptococcus cremoris* Wg2. Appl. Environ. Microbiol. **54:**43–49.
43. **Wohlrab, Y., and W. Bockelmann.** 1992. Purification and characterization of a dipeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus*. Int. Dairy J. **2:**354–361.
44. **Wohlrab, Y., and W. Bockelmann.** 1993. Purification and characterization of a second aminopeptidase (PepC-like) from *Lactobacillus delbrueckii* subsp. *bulgaricus* B14. Int. Dairy J. **3:**685–701.
45. **Wohlrab, Y., and W. Bockelmann.** 1994. Purification and characterization of a new aminopeptidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* B14. Int. Dairy J. **2:**409–427.
46. **Yamamoto, N., A. Akino, and T. Takano.** 1993. Purification and specificity of a cell-wall-associated proteinase from *Lactobacillus helveticus* CP790. J. Biochem. **5:**740–745.
47. **Zevaco, C., and J. C. Gripon.** 1987. Properties and specificity of a cell-wall proteinase from *Lactobacillus helveticus*. Lait **68:**393–408.