APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1997, p. 1284–1287 Vol. 63, No. 4
0099-2240/97/$04.00+0

Characterization of an Intracellular Oligopeptidase from *Lactobacillus paracasei*

R. O. TOBIASSEN,* T. SØRHAUG, AND L. STEPANIAK

Department of Food Science, Agricultural University of Norway, N-1432 Ås, Norway

Received 24 September 1996/Accepted 15 January 1997

An intracellular oligopeptidase from *Lactobacillus paracasei* Lc-01 has been purified to homogeneity by Fast Flow Q Sepharose, hydroxyapatite, and Mono Q chromatography. The molecular mass of the enzyme was determined to be 140 kDa by gel filtration and approximately 30 kDa by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and SDS-capillary electrophoresis. The pI of the enzyme was at pH 4.5. The enzyme expressed maximum activity at pH 8.0 and 40°C. Oligopeptidase activity on bradykinin was inhibited strongly by 1,10-phenantroline and EDTA and partly by *p*-chloromercuribenzoic acid but not by phosphoramidon or phenylmethylsulfonyl fluoride. Marked inhibition by β-casein fragment 58 to 72 was demonstrated. The enzyme showed neither general aminopeptidase nor caseinolytic activity, and it degraded only oligopeptides between 8 and 13 amino acids. The enzyme readily hydrolyzed the Phe-Ser and Pro-Phe bonds of bradykinin; the Phe-His bond of angiotensin I; the Pro-Gln, Gln-Phe, and Phe-Gly bonds of substance P; and the Pro-Tyr bond of neurotensin. Weak activity toward the Ala-Tyr and Pro-Ser bonds of α_{S1}-casein fragment 157 to 164, was observed. The N-terminal amino acid sequence of the oligopeptidase showed a high degree of homology to the lactacin B inducer from *Lactobacillus acidophilus*.

Mesophilic, homofermentative lactobacilli, mainly *Lactobacillus brevis*, *L. casei*, and *L. plantarum*, are common isolates of nonstarter lactic acid bacteria from high-quality cheeses (5). In particular, *L. paracasei* (formerly *L. casei*) is widely distributed (12). Compared to lactococci, the lactobacilli are more proteolytic (13) and may therefore be responsible for structure and flavor defects of cheeses (14). However, incorporation of selected lactobacilli into the cheese starter microflora may warrant a controllable maturation process and accelerate the cheese ripening (9).

Much is known about the proteolytic system of *Lactococcus* (18, 24); the information on *Lactobacillus* is more limited (2, 4). However, a number of similarities and differences between the proteolytic enzymes of lactobacilli and lactococci have been found by biochemical (4, 18), genetic (16, 17), and immunological (20, 23) characterizations.

Intracellular, lactococcal, noncaseinolytic oligopeptidases (PepO and PepF) from several lactococcal strains have been characterized, and the genes for one lactococcal PepO (16) and one lactococcal PepF (17) have been determined.

Characterization of intracellular oligopeptidases from lactobacilli is limited, but a 70-kDa endopeptidase from *L. delbrueckii* subsp. *bulgaricus*, similar to that of the lactococcal 70-kDa PepO (25), was characterized by Bockelmann (4).

The aim of this study was to search for oligopeptidase activity in *Lactobacillus*. Partial purification of intracellular extracts of *L. paracasei* Lc-01 resulted in five fractions with oligopeptidase activity. This paper reports the purification and characterization of one major oligopeptidase from this strain.

MATERIALS AND METHODS

Materials. Unless otherwise stated, all chemicals were of reagent grade and were obtained from Sigma Chemical Co., St. Louis, Mo. The casein fragments were synthesized at the Biotechnology Center of Oslo (University of Oslo, Oslo, Norway) with a 430A peptide synthesizer (Applied Biosystems, a division of Perkin-Elmer Corp., Foster City, Calif.).

Organism and preparation of the intracellular extract. *L. paracasei* Lc-01 was a gift from Chr. Hansen, Hørsholm, Denmark. The cells were grown in 10% reconstituted skim milk (Difco, Detroit, Mich.) at 37°C. The growth of the cultures was monitored by recording the absorbance at 480 nm of a 1:10 dilution of the culture in 0.2% (wt/vol) EDTA (trisodium salt) (6) and by observing the change in pH.

The cells were harvested from 4 liters of culture in the logarithmic growth phase (pH 5.1 to 5.2) by centrifugation (13,000 × *g* for 10 min at 4°C) after addition of 60 ml of 25% (wt/vol) trisodium citrate (Merck, Darmstadt, Germany) per liter and adjustment of the pH to 6.8 with 0.1 M NaOH (7). The supernatant was discarded, and the pellet was resuspended in 50 mM potassium phosphate buffer (pH 6.8) at 4°C (100 ml per liter of culture) and recentrifuged. The washing procedure was repeated once. The washed cells were then suspended in 62 ml of the same buffer, freeze-dried, and stored at −20°C.

The lyophilized cells (2.3 g) were suspended in 100 ml of spheroplast buffer (24% sucrose, 10 mM $MgCl_2$, 25 mM Tris buffer [pH 6.8]) containing lysozyme (1 mg/ml) (8) and incubated at 30°C for 30 min. After centrifugation (15,000 × *g* for 10 min at 20°C), the spheroplasts were suspended in 60 ml of 5 mM sodium phosphate buffer (pH 6.8). To ensure maximal liberation of intracellular material, the spheroplasts were sonicated (26) after being held on ice overnight. Cell breakage was observed by the decrease in optical density at 600 nm of 25 μl of the preparation dissolved in 1 ml of 5 mM sodium phosphate buffer (pH 6.8). After a 45-min treatment, the solution was centrifuged (48,000 × *g* for 30 min at 4°C), and the resulting supernatant was dialyzed against 50 mM sodium phosphate buffer (pH 6.8) at 4°C.

Purification of enzyme. The dialyzed intracellular extract was purified with fast protein liquid chromatography (FPLC) equipment (Pharmacia Biotechnology, Uppsala, Sweden). After separation on a Fast Flow Q Sepharose column (1.6 by 50 cm; Pharmacia) and a ceramic hydroxyapatite column (1.6 by 11.0 cm; Macro-Prep; Bio-Rad Laboratories, Hercules, Calif.) (26), the fractions that were most active on bradykinin were further purified on a prepacked Mono Q HR 5/5 column (Pharmacia) (21). Finally, the fraction that was most active on bradykinin was subjected to gel filtration on Superose 12 (1.6 by 50 cm; Pharmacia). The separation conditions were essentially as described by Stepaniak and Fox (21).

Enzyme assay. The reaction mixture consisted of 30 μl of 50 mM sodium phosphate buffer (pH 6.7), 6 μl of 5 mM bradykinin, 14 μl of H_2O, and 10 μl of enzyme solution. Incubation times were selected to give approximately 50% substrate reduction. After 20 to 30 min of incubation at 40°C, the reaction was stopped by addition of 60 μl of 1% trichloroacetic acid. The reaction mixture was centrifuged (12,000 × *g* for 3 min), and the supernatant was analyzed by capillary zone electrophoresis on BioFocus 3000 (Bio-Rad). The electrophoresis was run for 15 min on a coated capillary (25 μm by 24 cm; Bio-Rad) with 0.1 M high-performance electrophoresis (HPE) phosphate buffer (pH 2.5; Bio-Rad) at 20°C and 10 kV with detection at 220 nm. Between samples, the capillary was washed and equilibrated with 0.1 M NaOH for 3 min, H_2O for 3 min, and 0.1 M HPE phosphate buffer for 3 min. The activity was calculated from the rate of reduction of the substrate peak area.

Determination of molecular mass and pI. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing were carried out

* Corresponding author.

TABLE 1. Purification of an oligopeptidase from *L. paracasei* Lc-01

Purification step	Total amt of protein (mg)	Total activity (nmol/min)	Sp act (nmol/mg/min)	Purification (fold)	Yield (%)
Crude extract	119	5,400	45.5	1.0	100
Fast Flow Q Sepharose	31	1,400	45.2	1.0	26
Hydroxyapatite	2.86	813	284	6.2	15
Mono Q	0.87	163	187	4.1	3
Superose 12	0.09	61	678	15	1

with the Phast System (Pharmacia) as described by the manufacturer, using a ready-cast 20% SDS-PAGE gel and a ready-cast isoelectric focusing 3-9 gel (all from Pharmacia). The two gels were stained with silver nitrate and Coomassie blue, respectively, as specified by Pharmacia. Molecular mass and pI protein markers were from Pharmacia. The molecular mass of the enzyme was also determined by capillary electrophoresis (CE) with the CE-SDS protein kit (Bio-Rad).

Temperature and pH dependence of oligopeptidase activity. The effect of temperature on the oligopeptidase activity was measured in the range of 4 to 60°C. The substrate and buffer were equilibrated for 3 min at the test temperature before the addition of enzyme. The effect of the pH was determined in the range of pH 4 to 9 with universal buffer as described by Tan et al. (25).

Effects of chemical agents and β-casein fragment 58 to 72 on enzyme activity. The enzyme was preincubated with 2 mM each EDTA, phosphoramidon, 1,10-phenantroline, phenylmethylsulfonyl fluoride, *p*-chloromercuribenzoic acid, and β-casein fragment 58 to 72 [β-CN(f58-72)]. After incubation for 10 min at 40°C, sodium phosphate buffer (pH 6.7) and bradykinin were added, and the incubation was continued for 22 min. The reaction was stopped by the addition of trichloroacetic acid, and the residual activity was determined by capillary electrophoresis as described above.

Specificity studies. The specificity of the oligopeptidase was tested on several hormone peptides and on casein fragments (see Table 2). Enzyme (10 μl) was incubated with 1 mM substrate and 25 mM sodium phosphate buffer (pH 6.7) (total volume, 60 μl) for 3.5 to 4 h at 40°C. The reaction was stopped by the addition of 600 μl of 0.2% trifluoroacetic acid (Rathburn Chemicals Ltd., Walkerburn, Scotland), and the peptides were separated by reverse-phase (RP)–FPLC (Pharmacia) on a PepRPC HR 5/5 column (Pharmacia) equilibrated with 0.1% trifluoroacetic acid. The peptides were eluted with 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (Rathburn)-water (80:20 [vol/vol]) (solvent B). After elution for 6 min with solvent A, the concentration of solvent B was increased linearly from 0 to 50% within 33 min and from 50 to 100% within 15 min. Centers of peptide peaks detected at 214 nm were collected and freeze-dried for N-terminal amino acid sequence analysis.

Immunoblotting. Antibodies against PepO from *Lactococcus lactis* subsp. *lactis* MG1363 were raised as described by Tan et al. (25). Immunoblotting was performed in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) as specified by the manufacturer. An intracellular extract of *L. paracasei* Lc-01 was separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Bio-Rad), and developed with the horseradish peroxidase conjugate substrate kit (Bio-Rad).

Casein hydrolysis. Caseinolytic activity was determined by a modification of the method of Twining (27). The incubation mixture consisted of 10 μl of enzyme, 60 μl of 50 mM sodium phosphate buffer (pH 6.7), 60 μl of 0.5% fluorescent casein, and 3 μl of 10% NaN_3. The mixture was incubated at 40°C for 50 h. The reaction was stopped by the addition of 360 μl of trichloroacetic acid, and after 1 h at room temperature, the samples were centrifuged (12,000 × *g* for 5 min). A 400-μl portion of the supernatant was mixed with 2.6 ml of 500 mM Tris buffer (pH 8.5). The fluorescence was measured at 525 nm (emission) and 490 nm (excitation) with a Perkin-Elmer LS-5 luminescence spectrometer (Perkin-Elmer, Norwalk, Conn.).

General aminopeptidase activity. The aminopeptidase activity was determined with Leu-*p*-nitroanilide as the substrate (26). The enzyme was incubated for 24 h at 40°C.

Amino acid analysis. Freeze-dried enzyme was hydrolyzed in 6.0 M HCl in evacuated tubes for 24 h at 110°C. The amino acid composition was analyzed with an ABI 421 amino acid analyzer (Applied Biosystems) at the Biotechnology Center of Oslo.

N-terminal amino acid analysis. Automated Edman degradation was performed at the Biotechnology Center of Oslo with a 470A protein sequencer (Applied Biosystems). The phenylthiohydantoin derivatives of the amino acids were identified by high-pressure liquid chromatography (HPLC) on a 120A analyzer (Applied Biosystems).

Protein determination. The concentration of protein was determined by the Bio-Rad protein assay (Bio-Rad). Bovine serum albumin was used as the standard.

RESULTS

Enzyme purification. Fractionation of intracellular extract from *L. paracasei* Lc-01 on Fast Flow Q Sepharose, hydroxyapatite, and Mono Q resulted in the separation of five fractions that were active on bradykinin. The most active fraction, which was eluted from Mono Q at 0.25 M NaCl, was further characterized. A summary of the purification procedure is given in Table 1. The apparent low degree of purification, 15-fold, is

TABLE 2. Peptide bonds hydrolyzed in hormone peptides and peptides of caseinolytic origin by an oligopeptidase from *L. paracasei* Lc-01

Peptide	No. of amino acid residues	Sequence and peptide bonds hydrolyzed (arrows)	Identified peptides
Methionine enkephalin	5	NH[a]	
β-Casomorphin	7	NH	
α_{S1}-CN(f157–164)[b]	8	Asp-Ala-Tyr-Pro-Ser-Gly-Ala-Trp	Asp^{157}-Trp^{164} Tyr^{159}-Pro^{160}
Bradykinin	9	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	Arg^{1}-Phe^{5} Ser^{6}-Pro^{7} Phe^{8}-Arg^{9}
Angiotensin I	10	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu	Asp^{1}-Phe^{8}
Substance P	11	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met	Arg^{1}-Pro^{4} Phe^{7}-Phe^{8} Gly^{9}-Met^{11}
Neurotensin	13	Glu-Leu-Tyr-Glu-Asn-Lys-Pro-Arg-Arg-Pro-Tyr-Ile-Leu	Glu^{1}-Pro^{10} Glu^{1}-Leu^{13}
β-CN(f58–72)	15	NH	
β-CN(f194–209)	16	NH	
β-CN(f193–209)	17	NH	
Adrenocorticotropic hormone 1–17	17	NH	
α_{S1}-CN(f1–23)	23	NH	
Glucagon	29	NH	
Oxidized insulin β-chain	30	NH	
α_{S1}-CN(f165–199)	35	NH	

[a] NH, not hydrolyzed.
[b] Most of this peptide was not hydrolyzed.

caused by elimination of other bradykinin-hydrolyzing activities, conservative selection of active fractions, and inactivation during purification. The isolated enzyme was not stable when frozen; an 80% reduction in activity after 18 weeks at −20°C was observed. The purified enzyme was kept on ice throughout the process of characterization.

Molecular mass and isoelectric point. The enzyme was subjected to gel filtration on Superose 12, and the molecular mass was determined to be 140 kDa. Under reducing conditions, the molecular mass of the enzyme was estimated to be 30.5 to 35.5 kDa by CE-SDS. Silver-stained gels from SDS-PAGE showed a dominant band at 30 kDa. The pI of the enzyme was estimated to be 4.5 by isoelectric focusing.

Immunoblotting. No immunological reaction was observed between the crude intracellular extract of *L. paracasei* Lc-01 and antibodies raised against PepO from plasmid-free *Lactococcus lactis* subsp. *lactis* MG1363.

Temperature and pH dependence of oligopeptidase activity. The optimum temperature for bradykinin-hydrolyzing activity was found to be 40°C. At 4°C, the activity of the oligopeptidase was approximately 5% of the maximum activity. The optimum pH was 8.0, and no hydrolysis of bradykinin was detected below pH 5.5.

Substrate specificity. The enzyme showed no general aminopeptidase activity on Leu-*p*-nitroanilide or caseinolytic activity on fluorescent casein after a long incubation. Table 2 shows the activity of the enzyme on a selection of hormone peptides and peptides derived from casein. The enzyme was only active on α_{S1} casein fragment 157 to 164 [α_{S1}-CN(f157–164)], bradykinin, angiotensin I, substance P, and neurotensin, i.e., peptides containing 8 to 13 amino acid residues.

Effects of various chemical agents. The oligopeptidase was 100% inhibited by EDTA and 99% inhibited by 1,10-phenanthroline, both of which are inhibitors of metalloenzymes. The activity was not influenced by phosphoramidon, an agent known to inhibit many bacterial metalloendopeptidases. The isolated enzyme is hardly a serine enzyme, because no inhibition was observed after incubation with phenylmethylsulfonyl fluoride. The enzyme was partly inhibited (33%) by the thioproteinase inhibitor *p*-chloromercuribenzoic acid and was strongly inhibited (90%) by β-CN(f58–72). The peptide β-CN (f58–72) was originally isolated from Cheddar cheese and inhibited the 70-kDa lactococcal PepO (22).

Amino acid composition and N-terminal sequence. The amino acid composition of the purified oligopeptidase (Table 3) shows a high content of glutamine/glutamic acid (Glx), alanine, and leucine. The sulfur-containing amino acids, cysteine and methionine, are present at low concentrations.

Table 4 shows the sequence of the 19 N-terminal amino acids of the purified oligopeptidase. The sequence showed a high degree of homology to the N-terminal sequences of the lactacin B inducer from *L. acidophilus* (3) and triosephosphate isomerase from *Bacillus stearothermophilus* (1).

TABLE 3. Amino acid composition of the major oligopeptidase from *L. paracasei* Lc-01

Amino acid	Quantity[a] (pmol)	Molar ratio (%)	No. of amino acids (nearest integer)[c]
Asx	507	7	19
Glx	829	11	31
Ser	223	3	9
Gly	433	6	16
His	129	2	5
Arg	195	3	7
Thr	471	6	18
Ala	967	13	36
Pro	504	7	19
Tyr	198	3	7
Val	610	8	23
Met	98	1	4
Cys	29	<1	1
Ile	534	7	20
Leu	779	11	29
Phe	402	6	15
Lys	376	5	14
Trp	ND[b]		ND
Total	ca. 7,293		ca. 273

[a] Determined as described in Materials and Methods.
[b] ND, not determined.
[c] The nearest integer is calculated on the basis of a molecular mass of 30 kDa for the enzyme and an average molecular mass for an amino acid of 110 kDa.

DISCUSSION

Intracellular caseinolytic and noncaseinolytic endopeptidases, both monomers and oligomers, have been reported for *E. coli* (15), lactic acid bacteria (4, 24), and propionibacteria (26). This study indicates that the intracellular proteolytic system in *Lactobacillus* is complex and comprises several endopeptidases that are separable by ion-exchange and hydroxyapatite chromatography. These endopeptidases may be part of an intracellular protein turnover mechanism and a peptide degradation mechanism. The complexity may ensure alternative pathways for peptide degradation. This is supported by the observation of only an insignificant effect by the depletion of the 70-kDa PepO gene from *Lactococcus lactis* subsp. *cremoris* when grown in milk (16).

The oligopeptidase isolated from *L. paracasei* is apparently a metalloenzyme, similar to a number of noncaseinolytic lactococcal endopeptidases (17, 19, 25, 28, 29) and the slightly caseinolytic 40-kDa endopeptidase from *Propionibacterium* (26). Unlike the monomeric 70-kDa PepO from *Lactococcus lactis* subsp. *lactis* MG1363 (21) but similar to the 40-kDa monomeric endopeptidase from *Propionibacterium* (26), the apparently tetrameric *L. paracasei* oligopeptidase is not sensitive to phosphoramidon. However, in contrast to the *Propionibacterium* 40-kDa endopeptidase (26) and similar to the 70-kDa lactococcal PepO (22), the enzyme is inhibited by β-CN(f58–72).

The most striking feature of the *L. paracasei* oligopeptidase is the narrow restriction of its specificity by the length of the substrate. Other endo- and oligopeptidases of *Lactococcus* degraded oligopeptides containing between 5 and 30 residues (19, 25, 29). Characteristic of the *Lactobacillus* enzyme was restriction of the hydrolytic activity to peptides containing between 8 and 13 amino acids. In this respect, the enzyme is similar to the monomeric 70-kDa PepF from *Lactococcus lactis*, which degraded only peptides containing between 7 and 17 amino acid residues (17). The 70-kDa lactococcal PepO (19, 21, 25) hydrolyzed the Gly-Phe bond of methionine enkephalin (Tyr-Gly-Gly-Phe-Met) and had preferential specificity for X-Phe and X-Leu bonds of a number of oligopeptides. The oligopeptidase from *L. paracasei* showed less preference for these bonds and a different specificity on bradykinin from that of the 70-kDa lactococcal PepO (19), the lactococcal endopeptidase (LEP) II (29), or the 40-kDa *Propionibacterium* endopeptidase (26).

In contrast to the enzymes from *Lactococcus* and *Propionibacterium*, the *L. paracasei* oligopeptidase showed very little activity at the pH and temperature ranges that prevail during the ripening of cheeses. This, along with inability of the enzyme

TABLE 4. N-terminal amino acid sequence of the major oligopeptidase from *L. paracasei* Lc-01 isolated in the present study[a]

Protein	N-terminal amino acid sequence	Homology (%)
	1 5 10 15	
Oligopeptidase (present study) (*L. paracasei*)	M-R-T-P-F-I-A-G-N-W-K-M-N-K-N-P-K-E-T-	
Lactacin B inducer (*L. acidophilus*)	S-R-T-P-I-I-A-G-N-W-K-L-N-M-N-P-K-E-T-	79
Triosephosphate isomerase (*B. stearothermophilus*)	M-R-K-P-I-I-A-G-N-W-K-M-H-K-T-L-A-E-A-	63

[a] This sequence is compared with the N-terminal sequence of lactacin B inducer from *L. acidophilus* (3) and the N-terminal sequence of triosephosphate isomerase from *B. stearothermophilus* (1).

to degrade α_{S1}-CN(f1–23), indicates that it is less significant for cheese maturation than are the lactococcal PepO enzyme (25) or cell envelope-associated proteinase (10). However, chymosin and lactococcal cell envelope-associated proteinases release a number of short peptides from α_{S1}-casein and β-casein (11), which may be suitable substrates for the present enzyme. Thus, even if it is not the most important enzyme present, it may influence the final pattern of peptides in mature cheese.

The unique character of the *L. paracasei* oligopeptidase is indicated by its lack of homology to N-terminal fragments of lactococcal endopeptidases (16, 17, 19, 29) and by its immunological nonidentity with the lactococcal 70-kDa PepO. The significance of its homology to the lactacin B inducer from *L. acidophilus* (3) is questionable. With a molecular mass of 30 kDa, the enzyme may contain approximately 270 amino acid residues, and the sequence examined for homology represents only 7% of the total protein.

This study indicates that the family of endopeptidases from lactic acid bacteria is quite diverse and may encompass a subfamily of PepF-like oligopeptidases with specificity restricted to a narrow range of oligopeptides.

ACKNOWLEDGMENT

The financial support from the Norwegian Research Council is gratefully acknowledged.

REFERENCES

1. **Artavanis-Tsakonas, S., and J. I. Harris.** 1980. Primary structure of triosephosphate isomerase from *Bacillus stearothermophilus*. Eur. J. Biochem. **108:** 599–611.
2. **Atlan, D., C. Gilbert, B. Blanc, J. Frot-Coutaz, and R. Portalier.** 1993. Proteolytic system of lactobacilli, p. 31–37. *In* E.-L. Foo, H. G. Griffin, R. Möllby, and C.-G. Hedén (ed.), The lactic acid bacteria. Proceedings of the First Lactic Acid Bacteria Computer Conference. Horizon Scientific Press, Wymondham, England.
3. **Barefoot, S. F., Y.-R. Chen, T. A. Hughes, A. B. Bodine, M. Y. Shearer, and M. D. Hughes.** 1994. Identification and purification of a protein that induces production of the *Lactobacillus acidophilus* bacteriocin lactacin B. Appl. Environ. Microbiol. **60:**3522–3528.
4. **Bockelmann, W.** 1995. The proteolytic system of starter and non-starter bacteria: components and their importance for cheese ripening. Int. Dairy J. **5:**977–994.
5. **Bromme, M. C., D. A. Krause, and M. W. Hickey.** 1990. The isolation and characterization of lactobacilli from cheddar cheese. Aust. J. Dairy Technol. **45:**60–66.
6. **Cliffe, A. J., and B. A. Law.** 1985. Discontinuous polyacrylamide gel electrophoresis of cell wall proteinase from variants of *Streptococcus lactis*. J. Appl. Bacteriol. **58:**245–250.
7. **Coolbear, T., R. Holland, and V. L. Crow.** 1992. Parameters affecting the release of cell surface components and lysis of *Lactococcus lactis* subsp. *cremoris*. Int. Dairy J. **2:**213–232.
8. **Crow, V. L., R. Holland, and T. Coolbear.** 1993. Comparison of subcellular fractionation methods for *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. Int. Dairy J. **3:**599–611.
9. **El Soda, M. A.** 1993. The role of lactic acid bacteria in accelerated cheese ripening. FEMS Microbiol. Rev. **12:**239–252.
10. **Exterkate, F. A., A. C. Alting, and C. J. Slangen.** 1991. Specificity of two genetically related cell-envelope proteinases of *Lactococcus lactis* subsp. *cremoris* towards α_{S1}-casein-(1–23)-fragment. Biochem. J. **273:**135–139.
11. **Fox, P. F., T. K. Singh, and P. L. H. McSweeney.** 1995. Biogenesis of flavour compounds in cheese. Adv. Exp. Med. Biol. **367:**59–98.
12. **Hertel, C., W. Ludwig, B. Pot, K. Kersters, and K.-H. Schleifer.** 1993. Differentiation of lactobacilli occurring in fermented milk products by using oligonucleotide probes and electrophoretic protein profiles. Syst. Appl. Microbiol. **16:**463–467.
13. **Hickey, M. W., A. J. Hillier, and G. R. Jago.** 1983. Peptidase activities in lactobacilli. Aust. J. Dairy Technol. **38:**118–123.
14. **Martley, F. G., and V. L. Crow.** 1993. Interactions between non-starter microorganisms during cheese manufacture and ripening. Int. Dairy J. **3:**461–483.
15. **Maurizi, M. R.** 1992. Proteases and protein degradation in *Escherichia coli*. Experientia **48:**178–201.
16. **Mierau, I., P. S. T. Tan, A. J. Haandrikman, J. Kok, K. L. Leenhouts, W. N. Konings, and G. Venema.** 1993. Cloning and sequencing of the gene for a lactococcal endopeptidase, an enzyme with sequence similarity to mammalian enkephalinase. J. Bacteriol. **175:**2087–2096.
17. **Monnet, V., M. Nardi, A. Chopin, M.-C. Chopin, and J.-C. Gripon.** 1994. Biochemical and genetic characterization of PepF, an oligopeptidase from *Lactococcus lactis*. J. Biol. Chem. **269:**32070–32076.
18. **Pritchard, G. G., and T. Coolbear.** 1993. The physiology and biochemistry of the proteolytic system in lactic acid bacteria. FEMS Microbiol. Rev. **12:**179–206.
19. **Pritchard, G. G., A. D. Freebairn, and T. Coolbear.** 1994. Purification and characterization of an endopeptidase from *Lactococcus lactis* subsp. *cremoris* SK11. Microbiology **140:**923–930.
20. **Sasaki, M., B. W. Bosman, and P. S. T. Tan.** 1995. Immunological and electrophoretic study of the proteolytic enzymes from various *Lactococcus* and *Lactobacillus* strains. J. Dairy Res. **62:**611–620.
21. **Stepaniak, L., and P. F. Fox.** 1995. Characterization of the principal intracellular endopeptidase from *Lactococcus lactis* subsp. *lactis* MG1363. Int. Dairy J. **5:**699–713.
22. **Stepaniak, L., P. F. Fox, T. Sørhaug, and J. Grabska.** 1995. Effect of peptides from the sequence 58–72 of β-casein on the activity of endopeptidase, aminopeptidase, and X-prolyl-dipeptidyl aminopeptidase from *Lactococcus lactis* ssp. *lactis* MG1363. J. Agric. Food Chem. **43:**849–853.
23. **Tan, P. S. T., M.-P. Chapot-Chartier, K. M. Pos, M. Rousseau, C.-Y. Boquien, J.-C. Gripon, and W. N. Konings.** 1992. Localization of peptidases in lactococci. Appl. Environ. Microbiol. **58:**285–290.
24. **Tan, P. S. T., B. Poolman, and W. N. Konings.** 1993. Proteolytic enzymes of *Lactococcus lactis*. J. Dairy Res. **60:**269–286.
25. **Tan, P. S. T., K. M. Pos, and W. N. Konings.** 1991. Purification and characterization of an endopeptidase from *Lactococcus lactis* subsp. *cremoris* Wg2. Appl. Environ. Microbiol. **57:**3593–3599.
26. **Tobiassen, R. O., A. H. Pripp, L. Stepaniak, and T. Sørhaug.** 1996. Purification and characterization of an endopeptidase from *Propionibacterium freudenreichii*. J. Dairy Sci. **79:**2129–2136.
27. **Twining, S. S.** 1984. Fluorescein isothiocyanate-labeled casein assay for proteolytic enzymes. Anal. Biochem. **143:**30–34.
28. **Yan, T.-R., N. Azuma, S. Kaminogawa, and K. Yamauchi.** 1987. Purification and characterization of a substrate-size-recognizing metalloendopeptidase from *Streptococcus cremoris* H61. Appl. Environ. Microbiol. **53:**2296–2302.
29. **Yan, T.-R., N. Azuma, S. Kaminogawa, and K. Yamauchi.** 1987. Purification and characterization of a novel metalloendopeptidase from *Streptococcus cremoris* H61. Eur. J. Biochem. **163:**259–265.