Purification and Characterization of Intracellular Proteinase from *Lactobacillus casei* ssp. *casei* LLG

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ABSTRACT

The intracellular proteinase of *Lactobacillus casei* ssp. *casei* LLG was isolated in the cytoplasmic fraction with 0.05 M Tris-HCl buffer (pH 7.5). The enzyme was purified by the fast protein liquid chromatography system equipped with ion-exchange and gel filtration chromatographies. This proteinase comprised a single monomeric form and had a molecular weight of about 55 kDa and an isoelectric point near pH 4.9. The optimum pH and temperature for the enzyme activity were determined to be pH 6.5 and 37°C, respectively. The enzyme was inactivated by metal-chelating compounds (EDTA, 1,10-phenanthroline) and less affected by serine proteinase inhibitors (diisopropylfluorophosphate, phenylmethylsulfonyl fluoride). Proteinase activity was increased by Ca⁺⁺, Mn⁺⁺, and Co⁺⁺, and inhibited by Cu⁺⁺, Mg⁺⁺, and Zn⁺⁺. The activity of this enzyme to hydrolyze casein appeared to be more active on β -casein than α _{s1}-casein and κ -casein as monitored by polyacrylamide gel electrophoresis.

(**Key words:** *Lactobacillus casei*, proteinase, purification)

Abbreviation key: LAB = lactic acid bacteria, NSLAB = nonstarter LAB.

INTRODUCTION

In Cheddar cheese, the starter *Lactococcus* cells normally reach about 10⁹ cfu/g after salting stage. In our previous study and others (Lee et al., 1990a; Trépanier et al., 1992a, 1992b; Demarigny et al., 1996; 1990b; Roy et al., 1997; Beresford et al., 2001), the nonstarter lactic acid bacteria (NSLAB) population that was present at low concentrations (<10³/g) was increased to about

10⁸⁻⁹ cfu/g after about 3 to 4 mo. The NSLAB was always predominated by the *Lactobacillus casei* group (*Lb. casei*/*Lactobacillus paracasei*/*Lactobacillus rhamnosus*) in good quality of mature Cheddar, and they were judged to improve cheese flavor, but their contribution to cheese flavor and their origins are still unclear. It appears to be a general trend that *Lb. casei* group predominated in later stage of ripening, regardless of cheese types (in particular for semi- and hard type), and this flora is unlikely unique to each plant and each country (Lee et al., 1990a, 1990b; Linberg et al., 1996; Beresford et al., 2001).

Considering the very hostile environment of low temperature (5 to 6°C), redox potential, water activity, pH, and lactose, but high salt, it is intriguing how *Lb. casei* grows to such large numbers in cheese. Their energy sources and growth factors have been much debated. *Lactobacillus casei* seems to grow very well in starter cell debris, and their products released by autolysis (Thomas, 1987), peptides (Nath et al., 1973), and glycoproteins of milk fat (Williams and Banks, 1997). It has also been shown that NSLAB grow fast in Cheddar cheese (with a generation time of 8.5 d at 6°C), thus low levels of contamination will result in their dominance of total cheese flora (Jordan and Cogan, 1993). Randomly amplified polymorphic DNA was also used to detect the concentrations of NSLAB in Cheddar cheese, confirming the predominance of *Lb. paracasei* type after 4 to 8 wk of ripening (Fitzsimons et al., 1999).

Several studies have thus been carried out to accelerate Cheddar cheese with selected adjunct live *Lb. casei* or *Lactococcus lactis* cultures or shocked/lysis cultures (El Abboudi et al., 1991; Trépanier et al., 1992a, 1992b; McSweeney et al., 1994; Buist et al., 1998; Kang et al., 1998; Madkor et al., 2000).

During cheese ripening, evidence of autolysis and lysis of *Lb. helveticus* and *Lb. casei* also occurred naturally by the release of the intracellular enzymes (Dako et al., 1995; Kang et al., 1998; Valence et al., 1998). Thus proteolysis in cheese through both cell envelope-associated proteinase (PrtP or PrtH) and intracellular

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proteinase, along with more than 10 peptidases (PepN, PepX, PepO, PepQ, PepW, etc.), play a role in development of the desired texture, aroma, and flavor during cheese ripening (Courtin et al., 2002).

The cell-wall-associated proteinases have been reported from *Lactococcus lactis* ssp. *cremoris* AC1 (Geis et al., 1985), *Lactobacillus delbrueckii* ssp. *bulgaricus* (Kawai et al., 1999), *Lactococcus lactis* (Buis et al., 1998), and *Lactobacillus casei* (El Soda et al., 1986; Ezzat et al., 1988; Dhingra and Dutta, 2000). The cell-envelope proteinase genes have also been sequenced from *Lactococcus lactis*, *Lactobacillus paracasei*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii*, *Streptococcus pyogenes*, and *Streptococcus agalactiae* (Siezen, 1999). However, the data on the intracellular proteinases from lactic acid bacteria (LAB) are very limited (Ohmiya and Sato, 1975; Muset et al., 1989; Akuzawa and Okitani, 1995), and their functions are not well known.

Lactobacillus casei ssp. *casei* LLG expresses a high level of intracellular proteinases and aminopeptidases (Arora and Lee, 1994; Habibi-Najafi and Lee, 1994), the combined activity of these enzymes results in the production of cheese flavor compounds (Trépanier et al., 1992a, 1992b; Arora and Lee, 1994; Park et al., 1994; Arora et al., 1995; Christensen et al., 1999). Therefore, purification of the enzyme from LAB and characterization of their properties are considered to be indispensable for elucidation of the mechanisms involved in the processes of texture and flavor formation during cheese ripening and preparation of enzyme-modified cheese (Park et al., 1995; Habibi-Najafi and Lee, 1996).

The intracellular proteinase from *Lb. casei* ssp. *casei* LLG was thus purified to determine the specificity on the caseins and clarify the role of this enzyme for the production of accelerated-ripened cheese and enzyme-modified cheese.

MATERIALS AND METHODS

Chemicals and Materials

Azocasein, lysozyme from chicken egg white, and mutanolysin from *Streptococcus globisporus* ATCC 21553 were from Sigma Chemical (St. Louis, MO). Gels, stain dye, and molecular weight markers for the electrophoresis were supplied by Pharmacia (Baie d'Urfe, QC, Canada). All other chemicals were of analytical grade. Ultrafiltration membranes (Centriprep-30) were from Amicon (Beverly, MA). Chromatographic and PD-10 columns were obtained from Pharmacia.

Microorganism and Culture Conditions

Lactobacillus casei ssp. *casei* LLG used in this study was isolated from a mature Cheddar cheese and tenta-

tively identified by API 50 CHL (Merieux, St-Laurent, QC, Canada). Identification was further achieved by genetic techniques with RFLP and single-strand conformation polymorphism using PCR to amplify specific sequences of aminopeptidase (PepN) genes of *Lactobacillus rhamnosus* according to our previous method (Lee and Robert, 1997; Belanger, 1998).

Stock culture was maintained in sterile 10% (wt/vol) skim milk (Difco, Detroit, MI), and working culture was revived from frozen culture by 3 consecutive transfers in the same skim milk.

Preparation of Cell-Free Extract

The washed cells were resuspended in appropriate amount of 0.05 M Tris-HCl buffer (pH 7.5) and then disrupted by 3 passages through a French press cell at 18,000 psi (1 psi = 703.0696 kg/m²). The enzyme extract was centrifuged (15,000 × *g*, 35 min, 4°C) to eliminate cell debris, and the supernatant fraction was used as the intracellular crude enzyme.

Proteinase Assay

The proteinase activity conducted in replicate assays was measured using the chromogenic substrate, azocasein, according to the modified method of Beynon (1992). A portion (100 μL) of 2% (wt/vol) azocasein in 50 mM sodium phosphate buffer (pH 7.0) was mixed with 100 μL of enzyme in an Eppendorf tube. The mixture was incubated at 37°C for 16 h, as the enzyme activity was linear over a 16-h assay. The reaction was terminated by the addition of 500 μL of 12% (wt/vol) TCA solution and mixed thoroughly. After 15 min at room temperature, the mixture was centrifuged at 12,000 × *g* for 5 min. After 500 μL of supernatant was added to 500 μL of 1.0 M NaOH, the absorbance was measured at 440 nm against a blank without enzyme. One unit of proteinase activity was defined as the amount of the enzyme that results in an increase of 0.01 absorbance per hour at 440 nm.

Protein Determination

Protein content conducted in replicate assays was determined according to Smith et al. (1985). The bicinchoninic acid protein assay reagent supplied with the system (Pierce Chemical Ltd., IL) was used, with BSA as the standard. The protein concentrations in the fractions from the column were measured at 280 nm.

Purification of Enzyme

The active enzyme fractions during the purification were stored at 4°C in the presence of stabilizers (10%

glycerol, 100 mM $(\text{NH}_4)_2\text{SO}_4$, 1 mM dithiothreitol). The intracellular crude enzyme was fractionated by salting out with ammonium sulfate (40 to 80%) and centrifuged at $10,000 \times g$ for 20 min at 4°C . The precipitate was dissolved in 0.02 M Tris-HCl buffer (pH 8.0), and desalted by Sephadex G-25 gel filtration (PD-10 column, Pharmacia) previously equilibrated with the same buffer. A Pharmacia fast protein liquid chromatography system was used for the purification.

First ion-exchange chromatography. The desalted crude extract was applied to a preparative Mono Q (HR 16/10) column equilibrated with 0.05 M Tris-HCl buffer (pH 8.0). The enzyme was eluted with a stepwise gradient of NaCl (0 to 1.0 M) in the same buffer at a flow rate of 3 mL/min. Fractions (4 mL) were collected and tested for the activity. Fractions with the highest enzyme activities were pooled, concentrated by ultrafiltration using a Centriprep-30 membrane, and desalted.

Second ion-exchange chromatography. The concentrated enzyme fraction was applied to the second ion-exchange (Mono Q HR 5/5) column equilibrated with 0.02 M bis-Tris-HCl buffer (pH 6.4). The enzyme was eluted with a stepwise gradient of NaCl (0 to 1.0 M) in the same buffer at a flow rate of 0.75 mL/min, and 1.5-mL fractions were collected. The active fractions were pooled, concentrated by ultrafiltration, and desalted.

Chromatofocusing. The active fraction was applied to a chromatofocusing Mono P column (20 cm $\times$ 0.5 cm) after equilibration of the column with 20 mL of 0.02 M bis-Tris-HCl buffer (pH 6.0). The proteins were eluted with a linear pH gradient from 6.0 to 4.0 at a flow rate of 0.5 mL/min. The pH gradient was generated with a mixture of ampholytes (Polybuffer 7 to 4, Pharmacia), previously adjusted to pH 4.0 with 6 M HCl. The pH value of the fractions was immediately adjusted to near neutral pH with 0.2 M Tris-HCl buffer (pH 7.5) to minimize loss of enzyme activity. The polybuffer was removed from the active fractions by passage through a PD-10 column. The enzyme was concentrated using a Centricon-30 membrane.

Gel filtration chromatography. The enzyme was subjected to a gel filtration (Superose-12 HR 10/30) column equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). Elution was performed at a flow rate of 0.5 mL/min, and 1-mL fractions were collected. The active fractions were pooled and kept frozen at -40°C for further studies.

Hydrolysis of Caseins

Reaction mixture consisted of 4 volumes of different caseins (α_{s1} -, β -, and κ -CN) in 0.05 M phosphate buffer,

Figure 1. Purification of the intracellular proteinase from *Lactobacillus casei* ssp. *casei* LLG, protein concentration (—), enzyme activity (···), and NaCl or pH gradient (---). A) Mono Q HR 16/10, B) Mono Q HR 5/5, C) Mono P, and D) Superose 12 HR 10/30.

Table 1. Summary of purification steps of proteinase from *Lactobacillus casei* ssp. *casei* LLG.

Purification steps	Total Protein (mg)	Total Activity (U ¹)	Specific activity ² (U)	Yield (%)	Purification (fold)
Crude extract	671.63	3513	5.2	100	1
(NH ₄) ₂ SO ₄ ppt	516.64	3402	6.6	97	1.3
Ion exchange I	43.61	1758	41.3	50	7.8
Ion exchange II	6.40	823	128.6	23	24.7
Chromatofocusing	1.45	556	383.4	16	73.7
Gel filtration	0.79	317	401.3	9	77.2

¹One unit of enzyme activity is defined as the amount of enzyme that results in an increase of 0.01 absorbance per hour at 440 nm under the assay condition.

²Specific activity is defined as enzyme units per milligram of protein.

pH 7.0, and 1 volume of enzyme solution and incubated at 37°C. Samples (10 μ L) were taken at 0, 0.5, 2, 6, and 12 h. The protein samples were diluted (4:1) in sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.005% (wt/vol) bromophenol blue, 5% (vol/vol) 2- β -mercaptoethanol, and heated for 4 min at 95°C before applying to the gels.

Polyacrylamide Gel Electrophoresis

The purity of the enzyme preparation was examined by native and SDS-PAGE using the Phast System (Pharmacia). The SDS-PAGE was performed according to the method of Laemmli (1970). The molecular weight of the subunit of the purified enzyme was determined by SDS-PAGE using 12.5% homogeneous PhastGel. The protein samples were mixed 1:1 with sample buffer (20 mM Tris-HCl, pH 8.0) containing 2 mM EDTA, 5% SDS, 10% β -mercaptoethanol and 0.001% (wt/vol) bromophenol blue, boiled for 4 min and applied to the gels. The low molecular weight proteins were used as standards for the predicting of molecular mass of the pure protein. The gels were stained with Coomassie blue-R 250.

RESULTS

Purification of Enzyme

Cell-free extracts obtained from the cytoplasmic fraction of *Lb. casei* ssp. *casei* LLG were subjected to ion-exchange (Mono Q HR 16/10 and Mono Q HR 5/5) columns, chromatofocusing (Mono P), and finally gel filtration column (Superose 12 HR 10/30). The elution profiles during purification are shown in Figure 1.

The fractions eluted at 0.10 to 0.12 M NaCl gradient of Mono Q HR 16/10 ion-exchange chromatography were subjected to Mono Q HR 5/5 ion-exchange column. Then the fractions eluted at 0.16 to 0.18 M NaCl gradient of Mono Q 5/5 column were subjected to Mono P isoelectrofocusing column. The fractions eluted at 4.4 to 4.9 pH gradient of Mono P column were subjected again to Superose 12 HR 10/30 gel filtration column. The scheme of purification is summarized in Table 1.

The enzyme was purified through 4 consecutive purification steps and appeared as a single band on SDS-PAGE (Figure 2). The molecular weight of enzyme was estimated to be 55 kDa by SDS-PAGE and gel filtration.

Effects of pH and Temperature

The effects of pH and temperature on the proteinase activity of purified enzyme were determined with azocasein. The appropriate amount of enzyme and substrate prepared in 0.05 M acetate (pH 5.5 and 6.0), 0.05

Figure 2. SDS-PAGE of the purified intracellular proteinase of *Lactobacillus casei* ssp. *casei* LLG on 12.5 % polyacrylamide gel. Lane 1: molecular weight marker, Lane 2: purified enzyme.

Figure 3. Effect of pH on the activity of intracellular proteinase from *Lactobacillus casei* ssp. *casei* LLG.

M sodium phosphate (pH 6.5 to 7.0), 0.05 *M* Tris-HCl (pH 7.5 to 8.0) buffers were incubated at 37°C, and the activity was measured. The appropriate amount of enzyme and substrate prepared in 0.05 *M* phosphate buffer (pH 6.5) were incubated at different temperatures (25 to 55°C), and the activity was measured. The optimal pH was about 6.5, but at the pH less than 6.0 and higher than 7.5, the enzyme activity was sharply decreased (Figure 3). The optimal temperature of the purified enzyme was about 37°C (Figure 4). The relative activity at 25°C was less than 45%, and about 20% of the activity was retained at 55°C.

Figure 4. Effect of temperature on the activity of intracellular proteinase from *Lactobacillus casei* ssp. *casei* LLG.

Table 2. Effect of divalent cations and inhibitors on the proteinase activity.

Ion ¹	Relative activity (%) ²
Control	100
CaCl ₂	103
CoCl ₂	109
CuSO ₄	46
MnCl ₂	128
MgCl ₂	86
ZnCl ₂	84
DIFP	84
PMSF	81
EDTA	26
1,10-Phenanthroline	14
Iodoacetamide	94
Iodoacetic acid	92
<i>p</i> -Chloromercuribenzoate	44

¹1.0 mM final concentration.

²Mean value of replicates.

Effect of Divalent Cations and Inhibitors

The effect of divalent cations on the proteinase activity of purified enzyme was determined by preincubation with a final concentration of 1 mM of cations in 0.05 *M* sodium phosphate buffer (pH 6.5) for 30 min at 37°C before addition of substrate. The effect of inhibitors on the activity of purified enzyme was determined by preincubation with a final concentration of 0.1, 1.0, and 10 mM of inhibitors for 30 min at 37°C before addition of substrate. Inhibition was expressed as a percentage of the activity without effector (control). The enzyme activity was strongly inhibited by Cu⁺⁺, Mg⁺⁺, and Zn⁺⁺ at 1.0 mM, but Ca⁺⁺, Co⁺⁺, and Mn⁺⁺ had no inhibitory effect. The activity was almost inhibited after the proteinase was treated with EDTA and 1,10-phenanthroline, but not strongly inhibited by phenylmethylsulfonylfluoride and diisopropylfluorophosphate treatment. The sulfhydryl group reagents, iodoacetic acid, and iodoacetamide had no significant effect on the proteinase activity (Table 2).

Hydrolysis of Caseins

The actions of intracellular proteinase of *Lactobacillus casei* ssp. *casei* LLG on α_{s1} -CN, β -CN, and κ -CN were observed by SDS-PAGE. This enzyme hydrolyzed all caseins but seemed to be more active on β -CN than α_{s1} -CN and κ -CN (Figure 5). In particular, β -CN was almost completely hydrolyzed by incubation for 12 h with this intracellular proteinase.

DISCUSSION

The proteolytic system (peptidases and proteinases) of lactic acid bacteria used in cheese making plays an

Figure 5. SDS-PAGE on the action of intracellular proteinase from *Lactobacillus casei* ssp. *casei* LLG on α_{s1} -, β -, and κ -CN.

important role in the texture and flavor of the cheese, and it is well known that *Lactococcus* species are the most important starter used in cheese manufacture (Beresford et al., 2001). Despite the extensive works on the role of cell-envelope associated proteinase, the data on intracellular proteinase from lactic acid bacteria are limited.

The intracellular proteinase *Lb. casei* ssp. *casei* LLG after several steps of purification showed that the purified enzyme is a monomeric enzyme with the apparent molecular mass of 55,000 as estimated by SDS-PAGE. Intracellular proteinases from other lactic acid bacteria were reported that *Lc. lactis* ssp. *cremoris* had a molecular mass of 140 kDa (Ohmiya and Sato, 1975), and *Lc. lactis* ssp. *lactis* NCDO 763 (Muset et al., 1989), and IAM 1198 (Akuzawa and Okitani, 1995) had a molecular mass of about 93 and 12 kDa, respectively. The sizes of intracellular proteinase from LAB appeared to be different among strains, and their function may also be different in cell. Maximum activity of intracellular proteinase from *Lb. casei* ssp. *casei* LLG was obtained at 37°C and pH 6.5. The enzyme properties were pH 6.5 to 7.0 and 30°C for *Lc. lactis* ssp. *cremoris* (Ohmiya and Sato, 1975), pH 7.5 and 45°C for *Lc. lactis* ssp. *lactis* NCDO 763 (Muset et al., 1989), pH 5.5 to 6.0 and 30°C for *Lc. lactis* ssp. *lactis* IAM 1198 (Akuzawa and Okitani, 1995).

The enzyme was activated by Ca^{++} , Mn^{++} , and Co^{++} , but inhibited by Cu^{++} , Mg^{++} , and Zn^{++} that are in agreement with those of *Lc. lactis* ssp. *cremoris* (Ohmiya and Sato, 1975). This enzyme was also inactivated by metal chelating compounds (EDTA, 1,10-phenanthroline) and less affected by serine proteinase inhibitors (diisopropylfluorophosphate, phenylmethylsulfonylfluoride) and SH-groups reagents (iodoacetamide, iodoacetic acid). These results indicate that this proteinase is a metalloenzyme characteristic, which agrees with those of *Lc. lactis* ssp. *cremoris* (Ohmiya and Sato,

1975) and *Lc. lactis* ssp. *lactis* NCDO (Muset et al., 1989) but does not agree with that of *Lc. lactis* ssp. *lactis* IAM 1198 (Akuzawa and Okitani, 1995).

Lactobacillus spp. are the major portion of the NSLAB in some cheeses. During ripening of Cheddar cheese, the intracellular proteolytic enzymes of *Lactobacillus casei* appeared to play an important role in protein breakdown after the death and lysis of the cells. *Lactobacillus casei* has the peptidolytic, esterolytic, and proteolytic activities (Roy et al., 1997), thus the use of *Lactobacillus* as adjunct cultures has gained much attention recently in the production of accelerated ripened cheese and enzyme-modified cheese (Arora et al., 1995; Park et al., 1995; Habibi-Najafi and Lee, 1996).

This enzyme hydrolyzed all milk casein fractions, but hydrolyzed β -CN more preferentially. On the basis of degradation patterns of α_{s1} -, β - and κ -CN, 2 proteinase specificity classes may be possible as in Lactococci, but their comparisons are meaningless as they are unrelated enzymes. This proteinase probably involves later stage in the proteolysis of cheese curd after cell lysis. Intracellular proteinases are probably important in the turnover of denatured protein, activation of zymogens, and termination of newly synthesized proteins (Muset et al., 1989).

REFERENCES

- Akuzawa, R., and A. Okitani. 1995. Purification and characterization of a cysteine proteinase with low activation energy from *Lactococcus lactis* ssp. *lactis* IAM 1198. *J. Dairy Sci.* 78:2609–2616.
- Arora, G., and B. H. Lee. 1994. Purification and characterization of aminopeptidase from *Lactobacillus casei* subsp. *casei* LLG. *J. Dairy Sci.* 75:700–710.
- Arora, G., F. Cormier, and B. H. Lee. 1995. Analysis of odor-active volatiles in Cheddar cheese headspace by multidimensional GC/MS/Sniffing. *J. Agric Food Chem.* 43:748–752.
- Belanger, E. 1998. Genetic identification of the *Lactobacillus* species using PCR-based PepN sequences. M.Sc. Thesis, McGill University, Montreal, QC, Canada.
- Beresford, T. P., N. A. Fitsimons, N. L. Brennan, and T. M. Cogan. 2001. Recent advances in cheese microbiology. *Int. Dairy J.* 11:259–274.
- Beynon, R. J. 1992. Prevention of uncontrolled proteolysis. Pages 40–51 in *Protein Purification Methods: A Practical Approach*. IRL Press, New York, NY.
- Buist, G., G. Venema, and J. Kok. 1998. Autolysis of *Lactococcus lactis* is influenced by proteolysis. *J. Bacteriol.* 180:5947–5953.
- Christensen, J. E., E. G. Dudley, J. A. Pederson, and J. L. Steele. 1999. Peptidases and amino acid catabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* 76:217–246.
- Courtin, P., M. Nardi, U. Wegmann, V. Joutsjoki, J. C. Ogier, J. C. Gripon, A. Palva, B. Henrich, and V. Monnet. 2002. Accelerating cheese proteolysis by enriching *Lactococcus lactis* proteolytic system with *Lactobacillus* peptidases. *Int. Dairy J.* 12:447–454.
- Dako, E., M. El Soda, J.-C. Vuillemand, and R. E. Simard. 1995. Autolytic properties and aminopeptidase activities of lactic acid bacteria. *Food Res. Int.* 28:503–509.
- Demarigny, Y., E. Beuvier, A. Dasen, and G. Duboz. 1996. Influence of raw milk microflora on the characteristics of Swiss-type cheeses. I. Evolution of microflora during ripening and characterization of facultatively heterofermentative lactobacilli. *Lait* 76:371–387.

- Dhingra, J., and S. M. Dutta. 2000. Isolation, purification and characterization of the cell envelope associated proteinase of *Lactobacillus casei* 300. *Indian J. Dairy Sci.* 53:177–183.
- El Abboudi, M., S. Pandian, G. Trépanier, R. E. Simard, and B. H. Lee. 1991. Heat-shocked lactobacilli for the acceleration of Cheddar cheese ripening. *J. Food Sci.* 56:946–949 and 953.
- El Soda, M., M. J. Desmazeavd, D. Le Bars, and C. Zevaco. 1986. Cell-wall-associated proteinase in *Lactobacillus casei* and *Lactobacillus plantarum*. *J. Food Prot.* 49:361–365.
- Ezzat, N., M. El Soda, and H. El Shafei. 1988. The cell-bound proteinase system of *Lactobacillus casei*-purification and characterization. *Int. J. Food Microbiol.* 6:327–332.
- Fitzsimons, N. A., T. M. Cogan, S. Condon, and T. Beresford. 1999. Phenotypic and genotypic characterisation of non-starter lactic acid bacteria in matured Cheddar cheese. *Appl. Environ. Microbiol.* 65:3418–3426.
- Geis, A., W. Bockelmann, and M. Teuber. 1985. Simultaneous extraction and purification of a cell wall-associated peptidase and β -casein specific protease from *Streptococcus cremoris* AC1. *Appl. Microbiol. Biotechnol.* 23:79–84.
- Habibi Najafi, M. B., and B. H. Lee. 1994. Purification and characterization of x-prolyldipeptidyl peptidase from *Lactobacillus casei* ssp. *casei* LLG. *Appl. Microbiol. Biotech.* 42:280–286.
- Habibi-Najafi, M. B., and B. H. Lee. 1996. Bitterness in cheese: A Review. *Crit. Rev. Food Sci. Nutr.* 36:397–411.
- Johnson, J. A., M. R. Etzel, C. M. Chen, and M. E. Johnson. 1995. Accelerated ripening of reduced-fat Cheddar cheese using four attenuated *Lactobacillus helveticus* CNRZ-32 adjuncts. *J. Dairy Sci.* 78:769–776.
- Jordan, K. N., and T. M. Cogan. 1993. Identification and growth of non-starter lactic acid bacteria in Irish Cheddar cheese. *Irish J. Agric. Food Res.* 32:47–55.
- Kang, O. J., L. P. Vezinz, S. Laberge, and R. E. Simard. 1998. Some factors influencing the autolysis of *Lactobacillus bulgaricus* and *Lactobacillus casei*. *J. Dairy Sci.* 81:639–646.
- Kawai, Y., K. Tadokoro, R. Konomi, K. Itoh, T. Saito, H. Kitazawa, and T. Itoh. 1999. A novel method for the detection of protease and the development of extracellular protease in early growth stages of *Lactobacillus delbrueckii* ssp. *bulgaricus*. *J. Dairy Sci.* 82:481–485.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* 227:680–685.
- Lee, B. H., L. C. Laleye, R. E. Simard, M. H. Munsch, and R. A. Holley. 1990a. Influence of homofermentative lactobacilli cultures on the evolution of the microflora and soluble nitrogen compounds in Cheddar cheese. *J. Food Sci.* 55:391–397.
- Lee, B. H., L. C. Laleye, R. E. Simard, R. A. Holley, D. B. Emmons, and R. N. Giroux. 1990b. Influence of the homofermentative lactobacilli cultures on physico-chemical and sensory properties of Cheddar cheese. *J. Food Sci.* 55:386–390.
- Lee, B. H., and N. Robert. 1997. Overexpression of *Lactobacillus rhamnosus* aminopeptidase in *E. coli* (U.S patent file no. 60/025,172).
- Linberg, A. M., A. Christiansson, E. O. Rukke, T. Eklund, and G. Molin. 1996. Bacterial flora of Norwegian and Swedish semi-hard cheese after ripening, with special reference to *Lactobacillus*. *Neth. Milk Dairy J.* 50:563–572.
- Madkor, S. A., P. S. Tong, and M. El Soda. 2000. Ripening of Cheddar cheese with added attenuated adjunct cultures of lactobacilli. *J. Dairy Sci.* 83:1684–1691.
- McSweeney, P. L. H., E. M. Walsh, P. F. Fox, T. M. Cogan, F. D. Drinan, and M. Castelo-Gonzalez. 1994. A procedure for the manufacture of Cheddar cheese under controlled bacteriological conditions and the effect of adjunct lactobacilli on cheese quality. *Irish J. Agric. Food Res.* 33:183–192.
- Muset, G., V. Monnet, and J.-C. Gripon. 1989. Intracellular proteinase of *Lactococcus lactis* subsp. *lactis* NCDO 763. *J. Dairy Res.* 56:765–778.
- Nath, K. R., and R. A. Ledford. 1973. Growth response of *Lactobacillus casei* variety *casei* to proteolysis products in cheese during ripening. *J. Dairy Sci.* 16:710–715.
- Ohmiya, K., and Y. Sato. 1975. Purification and properties of intracellular proteinase from *Streptococcus cremoris*. *Appl. Microbiol.* 30:738–745.
- Park, S. Y., B. F. Gibbs, and B. H. Lee. 1995. Effect of crude enzyme of *Lactobacillus casei* LLG on water-soluble peptides of enzyme-modified cheese. *Food Res. Int.* 28:43–49.
- Roy, D., M. Pitre, L. Blanchette, L. Savoie, G. Belanger, P. Ward, and J. L. Maubois. 1997. Monitoring proteolysis and cheese juice composition during ripening of Cheddar cheese made from microfiltered milk. *Lait* 77:521–541.
- Siezen, R. J. 1999. Multi-domain, cell-envelope proteinases of lactic acid bacteria. *Antonie van Leeuwenhoek* 76:139–155.
- Smith, P. K., R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Geoke, B. J. Olson, and D. C. Klenk. 1985. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 150:76–85.
- Thomas, T. 1987. Cannibalism among bacteria found in cheese. *N.Z. J. Dairy Sci. Technol.* 22:215–219.
- Trépanier, G., M. El Abboudi, B. H. Lee, and R. E. Simard. 1992a. Accelerated maturation of Cheddar cheese: Microbiology of cheese supplemented with *Lactobacillus casei*. *J. Food Sci.* 57:345–349.
- Trépanier, G., M. El Abboudi, B. H. Lee, and R. E. Simard. 1992b. Accelerated maturation of Cheddar cheese: Influence of added lactobacilli and commercial protease on composition and texture. *J. Food Sci.* 57:898–902.
- Valence, F., R. Richoux, A. Thierry, A. Palva, and S. Lortal. 1998. Autolysis of *Lactobacillus helveticus* and *Propionibacterium freudenreichii* in Swiss cheese: First evidence by using species-specific lysis markers. *J. Dairy Res.* 65:609–620.
- William, A. G., and J. M. Banks. 1997. Proteolytic and other hydrolytic enzyme activities in non-starter lactic acid bacteria (NSLAB) isolated from Cheddar cheese manufactured in the United Kingdom. *Int. Dairy J.* 7:763–774.