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Enhancement of Proteolysis by a *Lactococcus lactis* Bacteriocin Producer in a Cheese Model System

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The accelerating effect exerted on proteolysis in cheese–curd slurries by the bacteriocin producer *Lactococcus lactis* IFPL 105, which is able to induce cell lysis in starter adjuncts with high peptidase activity, *Lactobacillus casei* subsp. *casei* IFPL731, and *Lactococcus lactis* subsp. *lactis* T1 has been studied. The optimum conditions for the use of the bacteriocin producer have been investigated to avoid late acidification by the starter and promote early cell lysis with the subsequent release of the intracellular peptidases and the acceleration of ripening without altering the cheese-making process. The induced cell lysis was of special importance in the case of *Lc. lactis* T1, because this strain possesses high peptidase activity and therefore can greatly influence the development of cheese flavor when added as an adjunct starter. In this study, it has been found that addition of *Lc. lactis* IFPL 105 does not interfere with proper acidification of the curd by the starter and increases the level of amino nitrogen correlated with early lysis of adjuncts, owing to the presence of bacteriocin.

Keywords: *Bacteriocin; lysis; proteolysis*

INTRODUCTION

For obvious reasons, it is important for the dairy industry to shorten cheese ripening times. Hydrolysis of casein by rennet and microbial proteinases and peptidases leads to the production of peptides and amino acids that are important for the development of cheese characteristics (Fox et al., 1996). Because microbial peptidases are located in the interior of the cell (Tan et al., 1992), bacterial lysis is, logically, crucial in favoring the access of enzymes to their substrates and hence accelerating cheese ripening (Chapot-Chartier et al., 1994; Wilkinson et al., 1994a). Nevertheless, a correct balance between lysed and intact cells must be achieved to attain the desired ripening in different cheese types (Crow et al., 1995a).

There have been a number of studies on the autolytic capacity of lactic acid bacteria [see reviews by Chapot-Chartier (1996) and Lortal et al. (1997)], and cloning and induced overproduction of the principal autolysin of *Lactococcus lactis* has been achieved (Buist et al., 1995, 1997). The effect of induced starter lysis on cheese ripening has been studied by using lysozyme (Law et al., 1976), by adding phage to the cheese vat (Crow et al., 1995b), by induction of phage in lysogenic strains (Feirtag and McKay, 1987), and by using heat-shocked cells (Ardö et al., 1988; Asensio et al., 1996) and high pressure (Yokoyama et al., 1994; Casal et al., 1996).

The use of bacteriocins to induce lysis of lactic acid bacteria was recently suggested (Morgan et al., 1995; Casla et al., 1996), and a strain that produces three narrow spectrum bacteriocins has been used as an adjunct culture to accelerate lysis of starter during Cheddar cheese ripening (Morgan et al., 1997). Studies have been carried out at our laboratory on a bacteriocin with a broad inhibitory spectrum including other LAB and pathogens (Casla et al., 1996). The inhibitory compound produced by *Lactococcus lactis* IFPL105 has been proved to be active against *Lactococcus lactis* subsp. *lactis* IFPL359, its variant (Lac$^-$ Prt$^-$), *Lactococcus lactis* subsp. *lactis* T1, and *Lactobacillus casei* subsp. *casei* IFPL731 (Martínez-Cuesta et al., 1997), all successfully used in cheese manufacture (Requena et al., 1992). Lytic response to the crude bacteriocin was more pronounced with mid-log phase cells of *L. lactis* T1. However, the use in cheese-making of bacteriocins or the microorganisms that produce them can cause alterations in the cheese-making process, such as delayed acidification of the curd with a concomitant increase in residual lactose (Fox et al., 1996).

This paper seeks to determine the optimum conditions for the use of a *Lactococcus* bacteriocin producer that could be used to accelerate ripening of semihard cheese without altering the cheese-making process, using microorganisms with high peptidase activity as bacteriocin-sensitive adjuncts. Cheese–curd slurries were used to accelerate the enzyme reactions involved in proteolysis of the cheese.

MATERIALS AND METHODS

Microorganisms. Microorganisms used in this study were *Lc. lactis* subsp. *lactis* IFPL359, its variant *Lc. lactis* subsp. *lactis* T1, which is lactose negative and has reduced proteolytic activity (Lac$^-$ Prt$^-$) (Requena and McKay, 1993), and *Lb. casei* subsp. *casei* IFPL731. All strains have been used previously as starter and adjuncts in the manufacture of semihard cheese (Requena et al., 1992; Rodríguez et al., 1996a, 1997).

The bacteriocin-producing microorganism (Bac$^+$), identified as *Lb. curvatus* IFPL105, its Bac$^-$ variant, and the characteristics of the bacteriocin produced were described in a previous paper (Casla et al., 1996). Further experiments, including intracellular protein profile by SDS–PAGE and PCR analysis using genus specific probes, have revealed that the strain should be reclassified as *Lc. lactis*, and it is therefore referred to hereafter as *Lc. lactis* IFPL105. It shows a narrow carbohydrate fermentation pattern and a Lac$^-$ phenotype. All strains were stored as frozen cultures at −80 °C in 100 g L^{-1} reconstituted skim milk.

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Cheese Model System. Cheese model curds were prepared from commercial pasteurized cow's skim milk, basically as described by Rodríguez et al. (1996b). The starter used to inoculate 6 L of milk, held at 30 °C, was an overnight culture of *Lc. lactis* IFPL359 (0.8% inoculum). A culture of *Lc. casei* IFPL731 that had been grown overnight in sterile skimmed milk, supplemented with 1% casitone (Difco, Detroit, MI), 0.5% glucose, 20 mg L^{-1} manganese sulfate, and 40 mg L^{-1} sodium formiate, was used as adjunct (0.5% inoculum). In addition, harvested cells from an overnight culture of the bacteriocin producer *Lc. lactis* IFPL105 (Bac^+, batch A) or the variant Bac^- (batch B) were added to the cheese vat to give a concentration of ~7 log cfu mL^{-1}. Another control (batch C) was made up only with *Lc. lactis* IFPL359 and *Lb. casei* IFPL731. The milk of all three batches (A−C) was incubated at 30 °C, and after incubation for 30 min, approximately 8 log cfu mL^{-1} of *Lc. lactis* T1 (Lac^- Prt^-), harvested at the mid-log phase of growth in M-17 broth (Oxoid, Basingstoke, U.K.) containing 5 g L^{-1} glucose (M17-G), was added to the batches along with 0.2 g L^{-1} $CaCl_2$ and 0.025 g L^{-1} animal rennet (Chr. Hansen's, Copenhagen, Denmark). When the coagulum was ready, it was cut, heated to 37 °C, and scooped into cylindrical molds. After pressing, the curds were left to reach pH 5.2, at which point they were divided into 150 g portions. These were homogenized aseptically in a Stomacher Lab Blender (Seward Medical, London, U.K.) by adding 60 mL of 4.5% NaCl in sterile water. A fourth cheese slurry system was carried out in the same conditions as batch C but with the addition of 3200 AU mL^{-1} of crude bacteriocin in the NaCl solution (batch D).

The resulting cheese slurries were placed in sterile glass containers and incubated at 12 °C. Samples were taken for analysis at 0, 1, 4, 7, 13, and 32 days of incubation and analyzed in triplicate.

Microbiological Analyses. Viable lactococcal counts in cheese−curd slurries were determined using bromocresol purple lactose indicator agar (BCPL) (McKay et al., 1970). After 20 h of incubation at 30 °C, Lac^+ colonies were yellow, whereas Lac^- colonies were white. *Lc. lactis* IFPL105 grew as white colonies surrounded by an inhibition halo after overlaying BCPL plates with a lawn of *Lc. lactis* IFPL359 as sensitive indicator in M-17 soft agar (0.7%) containing 5 g L^{-1} lactose (M17-L). *Lb. casei* IFPL731 was enumerated on MRS agar (Oxoid) containing 50 μg L^{-1} vancomycine (Sigma, St. Louis, MO) following 1 day of incubation at 30 °C. Total viable counts were enumerated on MRS agar after incubation of plates at 30 °C for 2 days.

Physicochemical Analyses. The pH was determined in a Metrohm Model 691 pH-meter (Metrohm Ltd., Herisau, Suiza) using 1 g of cheese slurry homogenized with 5 mL of distilled water. Total solids were dried to constant weight at 105 ± 2 °C for gravimetric determination. Fat was determined following International Dairy Federation standards (International Dairy Federation, 1986) and total protein by the Kjeldhal procedure (AOAC, 1975). Amino nitrogen was determined from the non-protein nitrogen fraction, as described by Kuchroo et al. (1983) by reaction with trinitrobenzenesulfonic acid (TNBS).

Enzyme Analyses. Release of the intracellular enzymes, X-prolyl-dipeptidyl-aminopeptidase (PepXP) and phospho-β-galactosidase, in cheese slurry extracts was used as a marker of cell lysis. The enzyme extract was obtained by vortexing 5 g of cheese slurries with 5 mL of 50 mM sodium phosphate buffer, pH 7.0. After centrifugation (10000*g*, 10 min at 4 °C), the supernatants were filtered (0.22 μm). In the reaction mixture, 100 μL of the filtrate were incubated for 150 min at 37 °C with 100 μL of 1 mM Arg-Pro-*p*-nitroanilide (Sigma) and 1 mM *o*-nitrophenyl β-D-galactopyranoside-6-phosphate (ONPG-6-P) (Sigma) in 50 mM phosphate buffer, pH 7.0; total volume was brought to 500 μL with phosphate buffer. The reaction was stopped by adding 500 μL of 0.5 M Na_2CO_3 at 4 °C, followed by centrifugation. The supernatants were tested for the release of *p*-nitroaniline and *o*-nitrophenol by measuring the increase in absorbance at 410 nm. Activity was expressed as micromoles of product released per minute and per milliliter of extract (units mL^{-1}).

Detection and Quantification of Bacteriocin. Bacteriocin produced by *Lc. lactis* IFPL105 in milk or MRS broth was titrated by serial 2-fold dilutions of the culture supernatants, as described by Casla et al. (1996), using *Lc. lactis* IFPL359 as the sensitive indicator strain. Bacteriocin activity in the cheese−curd slurries was determined in agar well plates by dispensing 50 μL of slurry samples in wells and overlaying with a lawn of the sensitive indicator grown in M17-L soft agar (0.7%). Crude bacteriocin extract was obtained after 40% ammonium sulfate precipitation of *Lc. lactis* IFPL105 supernatants as described elsewhere (Casla et al., 1996).

Statistical Analysis. Statistical study of results was performed using one-way analysis of variance to determine significant differences ($P < 0.05$) in total nitrogen, amino acid nitrogen, enzymatic activity, pH, total solids, and fat matter among slurries.

RESULTS AND DISCUSSION

Test conditions for model cheese slurries were chosen on the basis of prior studies on milk model systems subjected to a heat sequence treatment, simulating the cheese-making process but without renneting. In these previous experiments, addition to milk of the bacteriocin producer, *Lc. lactis* IFPL105, along with the starter culture *Lc. lactis* IFPL359 did not influence correct acidification. This can be explained by the delayed growth of the Bac^+ strain in milk owing to its Lac^- phenotype, which retarded bacteriocin production and allowed normal growth and acidification by the starter *Lc. lactis* IFPL359. On the contrary, when a crude bacteriocin extract (128 UA mL^{-1}) was added directly to the milk, a sharp decrease of starter cell viability (approximately 1.2 log units after 30 min and 3.7 log units after 3 h) occurred. A considerable lag in acidification with pH remaining at ~6.5 was also achieved. The sensitivity to bacteriocin lysis of the starter strain *Lc. lactis* IFPL359 and adjuncts *Lb. casei* IFPL731 and *Lc. lactis* T1 has been previously referred (Martinez-Cuesta et al., 1997), the latter being the most sensitive one. In the milk model systems subjected to a heat sequence treatment, we observed that addition of *Lc. lactis* T1 (Lac^- Prt^-) grown up to mid-logarithmic growth phase favored its lysis because this is the point of maximum sensitivity to the bacteriocin and further growth is limited to its Lac^- Prt^- phenotype.

In the present work, addition of *Lc. lactis* IFPL105 along with the starter culture allowed proper acidification and pH was the same in all curds, ~5.2 after pressing. Bacteriocin was detected in the medium at the point of homogenization, being not detected further during incubation of the curds. Moreover, the composition of the cheese slurries did not differ from one system to another; average values (percent of sample) were as follows: fat, 2.4 (±0.0); dry matter, 33.7 (±1.1); and protein, 25.3 (±1.0).

Figure 1 shows the microbial evolution of *Lc. lactis* IFPL359 (a), T1 (b), and *Lb. casei* IFPL731 (c) over 32 days of incubation at 12 °C in the various systems. Bacterial counts in batch A (Bac^+) over the experimental period were lower ($P < 0.05$) than in the controls [B (Bac^-) and C]. The results for batch D [165 UA (g of added bacteriocin)$^{-1}$] were comparable to control C and are therefore not shown. Also not shown are the results for *Lc. lactis* T1 in batch B (Bac^-), as they were indistinguishable from the counts of variant Bac^- of *Lc. lactis* IFPL105. The highest losses of cell viability were found at day 1 of incubation of batch A. The biggest

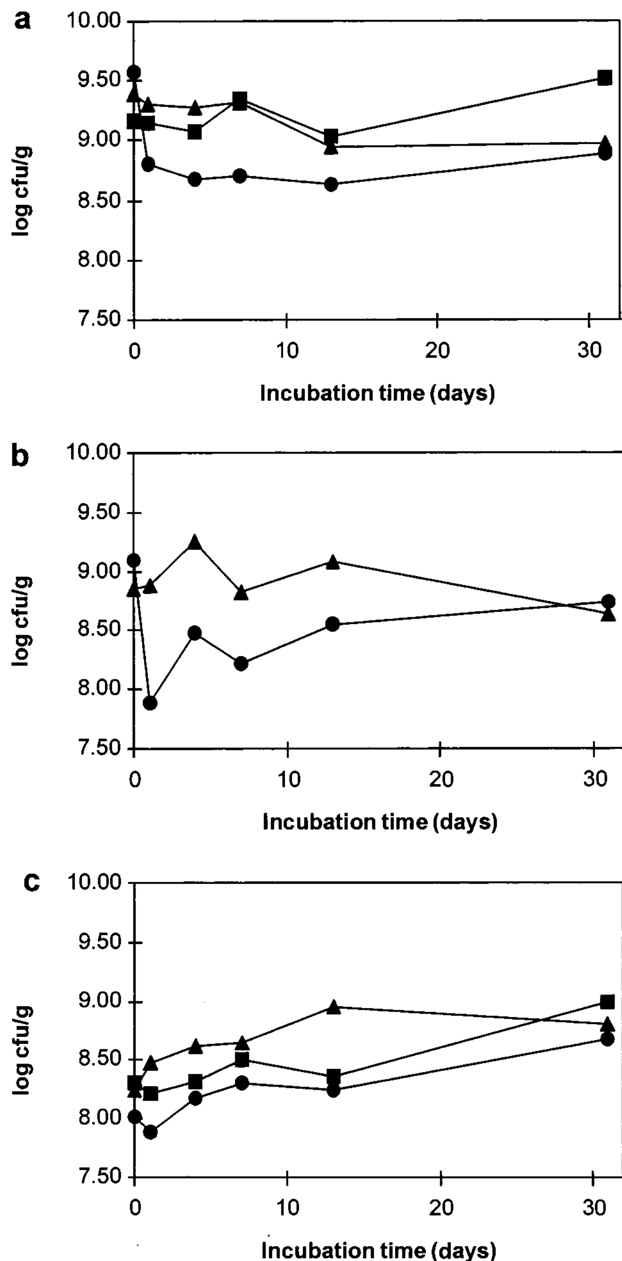

Figure 1. Evolution of *Lc. lactis* subsp. *lactis* IFPL 359 (a), its Lac⁻ Prt⁻ derivative strain T1 (b), and *Lb. casei* subsp. *casei* IFPL731 (c) in cheese−curd slurries supplemented with *Lc. lactis* IFPL 105 Bac⁺ (A, ●), its variant Bac⁻ (B, ■), and control (C, ▲).

decrease (1.2 log units) was for *Lc. lactis* T1 (added at the point of maximum sensitivity to the bacteriocin), as compared to losses of 0.77 and 0.21 log unit for *Lc. lactis* IFPL359 and *Lb. casei* IFPL731, respectively. The highest counts of *Lc. lactis* IFPL105 were found at the time of homogenization of the cheese curds (∼8.6 log cfu g^{-1}); they remained at this level throughout incubation. The positive results found about lysis of *Lc. lactis* T1 are of special relevance because this microorganism has been found to possess high peptidase activity and had influenced development of flavor when added as adjunct in the manufacture of low-fat cheese (Rodríguez et al., 1997).

Release of Intracellular Material. Although some spheroplast lysis due to enzymatic extraction under hypotonic conditions cannot be excluded (Wilkinson et al., 1994b), release of intracellular enzymes was used in the present work as an indicator of cell lysis during slurry incubation. Table 1 shows X-prolyl dipeptidyl aminopeptidase and phospho-β-galactosidase activities. Both were significantly higher ($P < 0.05$) in the cheese slurry made with *Lc. lactis* IFPL105 (Bac⁺) than in the controls in the first 4 days of incubation. The highest levels of intracellular activity were found in batch A (Bac⁺) at the outset of the study (0 and 1 day of incubation), corresponding with the highest levels of bacteriocin-induced loss of viability detected up to that stage. However, other studies (Crow et al., 1993; Chapot-Chartier et al., 1994; Morgan et al., 1997) have reported no immediate increase of free intracellular activity resulting from microbial loss of viability, which indicates that the two are not always related.

Table 1. Levels of Intracellular Enzyme Activities[a] in the Different Cheese−Curd Slurry Systems [A (bac⁺), B (bac⁻), and C (Control)] during Incubation

	incubation time					
	0 days	1 day	4 days	7 days	13 days	32 days
phospho-β-galactosidase						
A	0.46*	0.41*	0.14*	0.13*	0.11*	0.11*
B	0.36	0.31	0.11	0.09	0.06	0.04
C	0.36	0.19	0.11	0.11	0.07	0.03
PepXP						
A	4.06*	2.94*	3.52*	2.23	2.18*	2.00
B	3.44	2.37	3.13	2.35	2.07	1.68
C	2.82	2.35	3.11	2.40	1.76	2.16

[a] Phospho-β-galactosidase and X-prolyl dipeptidyl aminopeptidase (PepXP) activities are expressed as units per milliliter of cheese slurry extract. Values are the mean of three independent experiments. An asterisk indicates significant difference ($P < 0.05$) from values of batches B and C at the same incubation time.

In this study, both phospho-β-galactosidase and X-prolyl dipeptidyl aminopeptidase activity decreased during subsequent sample incubation, possibly due to the time-related inactivation reported by others (Wilkinson et al., 1994a). The reason for the differences between this case and the findings of Morgan et al. (1997), who measured increased intracellular activities during ripening, may be that in the latter case lysis was more gradual.

Evolution of Amino Nitrogen. The increase in amino nitrogen during cheese ripening is an indicator of proteolysis. It has also been correlated with enhanced sensitivity of starter strains to lysis (Chapot-Chartier et al., 1994).

Figure 2 shows the increase in amino nitrogen during incubation of the various cheese−curd slurry batches. There were significant differences ($P < 0.05$) between batch A (Bac⁺) and controls B and C after 7 days of incubation. After 1 month, the amino nitrogen content of the Bac⁺ system was 23 and 45% higher than that of batches B and C, respectively. In the Bac⁺ system, higher amino nitrogen correlated with higher microbial loss of viability and lysis owing to the presence of bacteriocin. This was due to greater release of intracellular microbial peptidases into the medium during cell lysis, which in turn increased the free amino acid content. These amino acids are essential precursors of compounds responsible for aroma (Fox and Wallace, 1997) and hence are a limiting factor in aroma development. Increasing their presence in the medium through induction of bacterial lysis is therefore considered a fundamental step in acceleration of proteolysis and development of the sensory characteristics of the cheese.

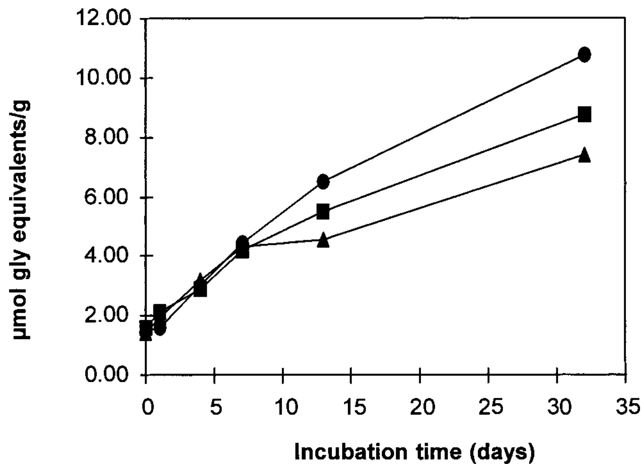

Figure 2. Evolution of amino nitrogen in cheese−curd slurries inoculated with *Lc. lactis* IFPL 105 Bac$^+$ (A, ●), its variant Bac$^-$ (B, ■), and control (C, ▲).

Conclusions. This study shows that use of the *Lc. lactis* IFPL105 bacteriocin producer in cheese−curd slurries causes a significant increase of amino nitrogen content and hence of proteolysis. Microbial lysis did not interfere with proper curd acidification by the starter due to optimization of conditions for use of the bacteriocin producer.

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