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# Release of the Cell-Envelope-Associated Proteinase of *Lactobacillus delbrueckii* Subspecies *lactis* CRL 581 Is Dependent upon pH and Temperature

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The cell-envelope-associated proteinase of *Lactobacillus delbrueckii* subsp. *lactis* CRL 581 (PrtL) has an essential role in bacterial growth and contributes to the development of the organoleptic properties of hard cheeses and to the release of bioactive health-beneficial peptides from milk proteins. In this study, the effect of environmental pH on PrtL production by *L. delbrueckii* subsp. *lactis* CRL 581 in a chemically defined medium and the influence of pH, temperature, and $Ca^{2+}$ ions on PrtL activity, stability, and release from the cell envelope were analyzed. The maximum PrtL activity levels were observed in the middle of the exponential growth phase, with the values at constant pH of 5.5 and 6.0 being higher than those observed at pH 4.5 and 5.0. At pH 4.5, PrtL remained mainly associated with the cell envelope, whereas at pH values of 5.5 or higher, approximately 40% of PrtL was found in the medium. In addition, the PrtL activity was stable for 24 h at 4 and 25 °C, and its release at 4, 25, and 40 °C was time-dependent. PrtL activity, stability, and release were independent of the presence of $Ca^{2+}$ ions in the medium. These results indicated that, at pH and temperature conditions found during the manufacture of hard cheeses, PrtL would remain active either bound to the cell or released in the supernatant contributing to the organoleptic characteristics and beneficial health effects of the fermented milk products.

**KEYWORDS: Proteinase; lactic acid bacteria; cheese; *Lactobacillus***

## INTRODUCTION

*Lactobacillus delbrueckii* subsp. *lactis* is a homofermentative thermophilic lactic acid bacterium (LAB) currently used in the industrial production of hard cheeses, such as Grana, Emmenthal, and Provolone. For its application, the fast-growing capacity of this microorganism in milk is crucial to enable a rapid acidification of the substrate (*1*). However, as with other LAB, *L. delbrueckii* subsp. *lactis* has a limited capacity to synthesize amino acids and is therefore dependent upon the use of exogenous nitrogen sources for optimal growth (*2*, *3*). The cell-envelope-associated proteinases (CEPs) of LAB are of major importance in this process, because they are responsible for the first step of casein degradation into peptides and amino acids (*4*). In addition to its essential role for bacterial growth in milk, CEPs also contribute to the development of texture and organoleptic characteristics of fermented milk products (*5*). Moreover, in the last few years, extensive scientific evidence has shown that certain CEPs can release bioactive peptides, which can contribute to promote health beyond the basic nutrition, during milk fermentation (*6*, *7*).

The proteolytic system of lactococci is the best documented among LAB (*4*, *8*). The CEPs of *Lactococcus lactis* SK11 and Wg2 have been characterized extensively both genetically and biochemically (*8*). These enzymes represent two of the several types of lactococcal CEPs, which have been distinguished on the basis of their specificity toward α- and β-caseins; the CEP from *Lc. lactis* Wg2 ($CEP_I$) showed a marked preference for β-casein, while that from *Lc. lactis* SK11 ($CEP_{III}$) used both α- and β-caseins (*8*). It has been observed that the release of these lactococcal CEPs from the cell surface occurs spontaneously in a $Ca^{2+}$-free buffer; removal of relatively weakly bound calcium in CEP initates a structural rearrangement in the proteinase domain, resulting in an enzyme that not only is susceptible to autoproteolytic release but also has a lower specific activity (*9*). In LAB-related serine proteinases (subtilases), four $Ca^{2+}$-binding sites have been described; the association of calcium ions to these sites affected the activity and thermal stability of the proteinases and protected the enzymes from autoproteolytic degradation (*10*). In addition, it is known that $Ca^{2+}$ ions stabilize the CEP after release (*11*). Lactobacilli CEPs have been less characterized, and little information about the role of calcium in the activity and stability of these enzymes is available.

*L. delbrueckii* subsp. *lactis* CRL 581 synthesizes a proteinase (PrtL) that is controlled by the peptide content of the growth medium (*6*). In addition, PrtL was able to release a series of potentially bioactive peptides (antihypertensive and phosphopeptides), which are encrypted within the precursor protein

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$\alpha_{s1}$- and $\beta$-caseins (*6*). Considering the industrial importance and the potential beneficial health properties of PrtL, studies on its activity and stability under conditions usually present in fermented milk products or cheese environment (e.g., low pH), could help to clarify the role of this enzyme in proteolysis. Therefore, in the present work, we describe the effect of environmental pH on PrtL production by *L. delbrueckii* subsp. *lactis* CRL 581 in a chemically defined medium. Also, the influence of temperature, pH, and calcium ions on PrtL activity, stability, and release from the cell envelope was analyzed.

## MATERIALS AND METHODS

**Microorganisms, Media, and Growth Conditions.** *L. delbrueckii* subsp. *lactis* CRL 581, isolated from a homemade Argentinian hard cheese, was obtained from the culture collection of CERELA (San Miguel de Tucumán, Argentina). In this study, a previously formulated minimal defined medium (MDM) containing 5 mM $CaCl_2$ (MDM−Ca) was used (*3*). MDM−Ca (pH 6.5) was prepared from concentrated individual stock solutions, which were stored at 4 °C after filtration, except for the cysteine solution, which was freshly prepared. All amino acids, vitamins, purines, pyrimidines, and inorganic salts used were of analytical grade (Sigma Chemical Co., St. Louis, MO). Medium and stock solutions were sterilized by filtration through a cellulose acetate membrane (0.20 $\mu$m pore size; Sartorius AG, Göttingen, Germany).

Working culture of *L. delbrueckii* subsp. *lactis* CRL 581 was propagated twice in MRS broth (Biokar Diagnostics, France) at 40 °C for 16 h. To eliminate carryover nutrients, cells were harvested by centrifugation at 8000*g* for 15 min, washed twice in sterile 0.85% (wt/vol) saline, and resuspended in this solution to the original volume. This cell suspension was used to inoculate the media of different pH values at an initial optical density at 560 nm ($OD_{560}$) of 0.1.

Controlled pH batch cultures were carried out in a 650 mL fermentor (New Brunswick Scientific Co., Edison, NJ). Agitation was applied at 100 rpm. The temperature was maintained at 40 °C, and the pH was kept automatically at 4.5, 5.0, 5.5, or 6.0 with either sterile 1 M HCl or $NH_4OH$. The inoculum was prepared as stated before and added to the fermentation vessel until an $OD_{560}$ of 0.1 was reached. Fermentations were allowed to proceed for 24 h; samples were aseptically withdrawn at several time intervals from the fermentation vessel and immediately cooled on ice to determine cell growth and proteinase activity. Bacterial growth was monitored by measuring the $OD_{560}$. The specific growth rate ($\mu_{max}$) was calculated from the slope of a semi-logarithmic plot of $OD_{560}$ versus time (h). Microbial growth was expressed as $\ln x/x_0$ versus time, where $x$ means $OD_{560}$ at time $t$ and $x_0$ means initial $OD_{560}$.

**Proteinase Activity Assay.** Cells grown in different media were harvested by centrifugation (10000*g*, 10 min, 4 °C), washed twice with saline supplemented with 10 mM $CaCl_2$, and resuspended to a final $OD_{560}$ of approximately 10 in 100 mM sodium phosphate (pH 7.0). The proteinase activity was measured with the chromogenic substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide (S-Ala; Sigma) as described by Exterkate (*12*). One unit of proteinase was defined as the amount required to liberate 1 nmol of nitroaniline/min; specific activity was expressed as proteinase units per milligram of protein. The protein concentration was determined using a protein assay according to the instructions of the manufacturer (Bio-Rad Laboratories, Richmond, CA). The level of cell lysis was determined by following the release of either lactate dehydrogenase (LDH) according to the method of Thomas (*13*) or lysyl-aminopeptidase (PepN) using L-lysine *p*-nitroanilide as the substrate as described previously (*14*).

**Influence of pH and Calcium on PrtL Activity and Stability.** Cells grown on MDM−Ca without pH control were harvested by centrifugation (10000*g*, 10 min, 4 °C) at the exponential growth phase, washed 3 times with saline solution, and resuspended to a final $OD_{560}$ of approximately 10 in the following buffers: 50 mM sodium acetate (pH 4.5, 5.0, 5.5, and 6.0) and 50 mM sodium phosphate (pH 6.5, 7.0, 7.5, and 8.0). The samples, incubated at 40 °C, were aseptically withdrawn at 0, 4, and 24 h and centrifuged (10000*g*, 10 min, 4 °C). The proteinase activity was measured in the supernatant and in the cell pellet (previously resuspended in 50 mM sodium phosphate at pH 7.0) at optimal conditions (*6*). Briefly, the assay mixture, containing 50 mM sodium phosphate buffer (pH 7.0), 1.5 M NaCl, 2 mM S-Ala, and 30 $\mu$L of the supernatant or cell pellet, was incubated at 40 °C for 10 min. The reaction was stopped by adding 25% acetic acid, and the samples were centrifuged (10000*g* for 5 min). The released nitroaniline was measured at 410 nm using a VERSAmax microplate reader (Molecular Devices Corp., Sunnyvale, CA). PrtL activity was expressed as a percentage of remaining enzyme activity with respect to the control (PrtL activity of whole cells determined under optimal conditions at initial time). The influence of calcium ions on PrtL activity was tested after adding 10 mM $CaCl_2$ to each buffer.

**Casein Hydrolysis.** Whole cells and the supernatants obtained as described above were mixed with 5 mg/mL of α- and $\beta$-caseins (Sigma) previously dissolved in 100 mM sodium phosphate (pH 7.0) at a ratio of 1:1. The resulting mixtures were incubated at 40 °C for 3 h and centrifuged (10000*g*, 10 min, 4 °C), and the supernatants were analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) as described previously (*15*). Protein bands were visualized by silver staining (Bio-Rad).

## RESULTS AND DISCUSSION

**Effect of pH on PrtL Production.** To determine the effect of pH on PrtL production, *L. delbrueckii* subsp. *lactis* CRL 581 was cultured in MDM−Ca at constant pH values of 4.5, 5.0, 5.5, or 6.0. At constant pH values of 5.0, 5.5, and 6.0 similar specific growth rates ($\mu_{max}$ = 0.42 $h^{-1}$) were observed (**Figure 1**); PrtL production increased concomitantly with the cell growth until the middle of the exponential growth phase and then decreased gradually until early stationary growth phase, with the enzyme being active even after 24 h of incubation. The PrtL activity levels produced by CRL 581 at pH 5.5 and 6.0 were higher than those observed at pH 5.0 (**Figure 1**). In contrast, at constant pH of 4.5, *L. delbrueckii* subsp. *lactis* CRL 581 showed a reduced growth rate ($\mu_{max}$ = 0.15 $h^{-1}$) and the PrtL activity was also scarce throughout the fermentation period (**Figure 1**). These results indicated that the PrtL production was affected by the microbial growth phase and the external pH.

**Effect of Calcium Ions and pH on PrtL Activity and Stability.** The most widely used method for releasing CEPs from LAB cells, particularly from lactococcal strains cells, consists of washing the cells in $Ca^{2+}$-free buffer. The removal of $Ca^{2+}$ from the CEP of *Lc. lactis* SK11 conduced also to a decrease in its specific activity and to a drastic reduction in thermal stability; thus, no activity at 25 °C and pH 6.5 could be measured (*9*). Althought the consequences of $Ca^{2+}$ removal were less drastic for the CEP activity and stability of *Lc. lactis* Wg2, the autoproteolytic release of this enzyme was more efficient than that of SK11 CEP (*9*). In this study, the release of PrtL after 4 h of incubation of *L. delbrueckii* subsp. *lactis* CRL 581 in buffer with or without 10 mM $CaCl_2$ was observed (**Table 1**). On the other hand, PrtL activity levels of *L. delbrueckii* subsp. *lactis* CRL 581 grown on MDM−Ca determined in the absence of $Ca^{2+}$ or with 10 mM $Ca^{2+}$ at pH 7.0 were similar [40.3 and 39.8 nmol $min^{-1}$ (mg of protein)$^{-1}$, respectively]. Furthermore, the addition of calcium ions to the incubation buffer of different pH values produced no change on PrtL activity in either the cell pellet or the supernatant. These results showed that the PrtL activity, release, and stability were not affected by the presence of calcium ions.

The maximum value of PrtL activity observed during growth at constant pH of 5.5 or 6.0 was similar to that obtained in the middle of the exponential growth phase under noncontrolled pH conditions (**Figure 1**). Nevertheless, the values of PrtL activity under free pH conditions after 24 h of incubation were about 3 times lower to those obtained at controlled pH of 5.5 or 6.0 (**Figure 1**), possibly because of the exposure of the enzyme to a pH value of about 4.2 for more than 12 h. The pH value of MDM−Ca after incubation of *L. delbrueckii* subsp. *lactis* CRL

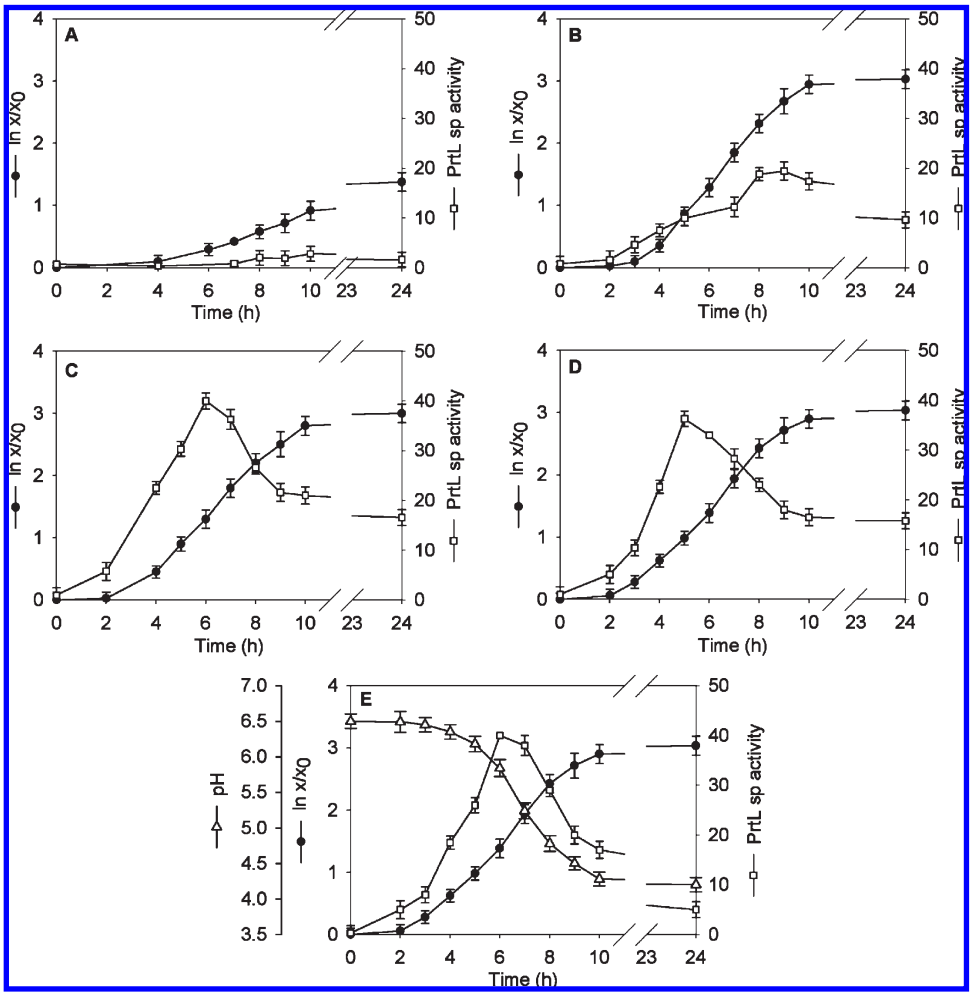


**Figure 1.** Growth and PrtL specific activity of *L. delbrueckii* subsp. *lactis* CRL 581 grown in MDM−Ca at pH (A) 4.5, (B) 5.0, (C) 5.5, and (D) 6.0 and (E) at uncontrolled pH. Error bars show standard deviations from three independent experiments.

**Table 1.** Effect of pH on the Stability of the Cell-Envelope-Associated and Released PrtL Incubated at 40 °C in $Ca^{+2}$-free Buffers after 4 and 24 h[a]

| | 4 h of incubation | | 24 h of incubation | |
|---|---|---|---|---|
| pH | cell-bound PrtL activity (%)[b] | released PrtL activity (%) | cell-bound PrtL activity (%) | released PrtL activity (%) |
| 4.5 | 64.46 ± 3.1 | 4.00 ± 0.3 | 32.26 ± 1.8 | 7.59 ± 0.5 |
| 5.0 | 69.93 ± 3.6 | 10.12 ± 0.6 | 37.53 ± 1.6 | 10.47 ± 0.6 |
| 5.5 | 68.40 ± 3.4 | 11.50 ± 0.7 | 39.63 ± 2.0 | 12.37 ± 0.3 |
| 6.0 | 51.41 ± 2.3 | 28.59 ± 1.9 | 34.10 ± 1.5 | 17.80 ± 0.6 |
| 6.5 | 54.81 ± 2.8 | 31.15 ± 1.6 | 22.20 ± 1.1 | 28.70 ± 1.2 |
| 7.0 | 54.58 ± 2.5 | 31.43 ± 1.5 | 21.73 ± 1.2 | 30.59 ± 1.7 |
| 7.5 | 55.51 ± 2.2 | 31.97 ± 1.7 | 24.51 ± 1.3 | 30.31 ± 1.4 |
| 8.0 | 56.06 ± 2.9 | 33.98 ± 2.3 | 23.38 ± 1.8 | 32.15 ± 1.5 |

[a] Values are the means ± standard deviations from three independent experiments. The values of PrtL activity after 4 and 24 h of incubation in $Ca^{2+}$-free buffers were similar to those observed in buffer containing 10 mM $CaCl_2$. [b] Activity is expressed as percentages of PrtL activity with regard to untreated cells measured at zero time.

581 at 40 °C under free pH conditions for 9 h was about 4.3, remaining approximately constant until the end of 24 h of fermentation (**Figure 1**). Because low pH values could affect PrtL activity and/or stability, the effect of pH on both properties was determined. Cells harvested in the middle of the exponential growth phase in MDM−Ca without pH control were incubated at 40 °C in $Ca^{2+}$-free buffers of different pH values for 4 and 24 h. Then, the samples were centrifuged, and the residual

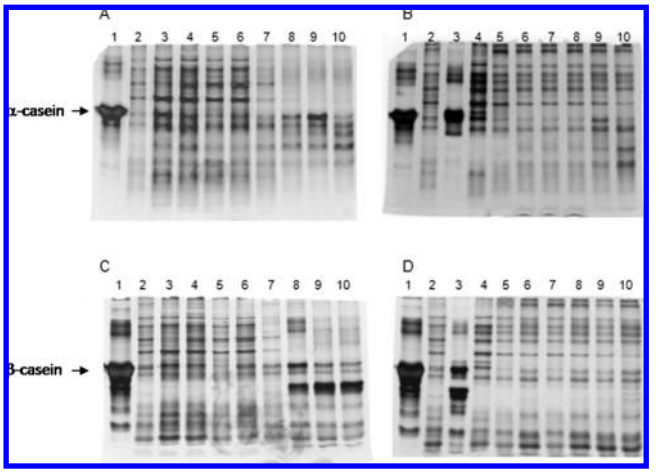


**Figure 2.** Hydrolysis of α-casein (A and B) and β-casein (C and D) by the cell-envelope-associated PrtL (A and C) and the released PrtL (B and D). Cell suspensions of *L. delbrueckii* subsp. *lactis* CRL 581 were preincubated during 4 h at 40 °C at different pH values. Lane 1, starting substrate (control); lane 2, samples without substrate; lane 3, pH 4.5; lane 4, pH 5.0; lane 5, pH 5.5; lane 6, pH 6.0; lane 7, pH 6.5; lane 8, pH 7.0; lane 9, pH 7.5; and lane 10, pH 8.0.

PrtL activity was measured in the supernatant (released PrtL) and in the cell pellet (cell-envelope-associated PrtL) by determining

**Table 2.** Stability at 4, 25, and 40 °C of the Cell-Bound and Released PrtL at pH 6.5 after Incubation at Different Periods[a]

| | 4 °C | | 25 °C | | 40 °C | |
|---|---|---|---|---|---|---|
| incubation time (h) | cell-bound PrtL activity (%)[b] | released PrtL activity (%) | cell-bound PrtL activity (%) | released PrtL activity (%) | cell-bound PrtL activity (%) | released PrtL activity (%) |
| 2 | 73.1 ± 3.9 | 27.2 ± 1.4 | 69.0 ± 3.2 | 32.2 ± 1.6 | 67.0 ± 3.1 | 30.1 ± 1.7 |
| 4 | 62.2 ± 2.9 | 37.4 ± 1.8 | 56.3 ± 2.8 | 36.1 ± 1.8 | 54.8 ± 2.8 | 31.1 ± 1.4 |
| 6 | 59.5 ± 3.0 | 40.6 ± 2.1 | 52.7 ± 2.3 | 40.3 ± 1.9 | 48.0 ± 2.3 | 33.0 ± 1.5 |
| 8 | 55.4 ± 2.6 | 43.8 ± 2.3 | 39.6 ± 2.0 | 40.2 ± 2.0 | 35.0 ± 1.9 | 33.4 ± 1.6 |
| 24 | 50.3 ± 2.4 | 48.5 ± 2.4 | 38.4 ± 1.7 | 40.4 ± 2.1 | 22.2 ± 1.1 | 28.7 ± 1.2 |

[a] Values are the means ± standard deviations from three independent experiments. The values of PrtL activity after 4 and 24 h of incubation in $Ca^{2+}$-free buffers were similar to those observed in buffer containing 10 mM $CaCl_2$. [b] Activity is expressed as percentages of PrtL activity with regard to untreated cells measured at zero time.

their action on the chromogenic substrate S-Ala (**Table 1**) as well as by SDS−PAGE, using α- and β-caseins as substrates (**Figure 2**).

After 4 h of incubation, more than 80% of PrtL activity were retained in both the cell pellet and the supernatant in a range of pH from 5.0 to 8.0, while only 68% of the original activity in both fractions was observed at pH 4.5 (**Table 1**). From this later value, about 94% remained attached to the cell envelope, while at pH values higher than 5.5 approximately 60% of the total PrtL activity was cell-attached (**Table 1**). As stated above, similar results were obtained after incubating the cells in the presence of 10 mM $Ca^{2+}$-supplemented buffer (data not shown).

The profile of α- and β-casein degradation observed for the released and cell-bound PrtL was similar. Cell-bound PrtL exposed during 4 h at pH of 4.5 hydrolyzed completely α- and β-caseins, while the breakdown of these substrates by the released PrtL was, as expected, lower (**Figure 2**). On the contrary, at pH higher than 5.0, both the released and the cell-bound PrtL degraded completely α- and β-caseins (**Figure 2**).

At 24 h of incubation, the residual PrtL activity values in the pH range of 5.0−8.0, in both the cell pellet and the supernatant, were approximately 50% of the initial PrtL activity levels (**Table 1**). PrtL activity detected in both fractions was dependent upon the pH of the medium. Because cell lysis was not observed throughout the incubation period, proteinase activity detected in the supernatant was ascribed to the released PrtL. At pH 6.5 or higher, the proteinase activity could be detected mainly in the supernatant, while the cell-envelope-associated PrtL activity was mainly detected at pH 6.0 or lower (**Table 1**). These results could explain the lower PrtL activity found after 24 h of incubation in controlled pH conditions of pH 5.0, 5.5, and 6.0. On the other hand, the low residual PrtL activity values observed at pH 4.5 indicated that the activity of PrtL, either released or attached, was sensitive to this pH value (**Table 1**). This fact may explain the reduced PrtL activity detected after 24 h of incubation of *L. delbrueckii* subsp. *lactis* CRL 581 grown under free pH culture conditions (**Figure 1**).

Finally, to analyze the effect of temperature on the liberation and stability of PrtL, the activity of this enzyme at 4, 25, and 40 °C and constant pH of 6.5 at different times of incubation was evaluated (**Table 2**). After 24 h of incubation at 4 °C, about 50 and 48% of PrtL activity were found associated with the cell pellet (cell-bound PrtL) and the supernatant (released PrtL), respectively (**Table 2**). On the other hand, the supernatant residual activity after 24 h of incubation at 25 and 40 °C was about 40 and 29%, respectively, while the values of the cell-associated PrtL activity were about 38 and 22%, respectively. These data demonstrated that the enzyme was more stable at 4 °C than at 25 or 40 °C. With the increase of the incubation time, the values of PrtL activity in the cell pellet decreased and the liberation of the enzyme was higher at any temperature (4, 25, or 40 °C; **Table 2**). These results showed that PrtL was released into the supernatant at the three temperatures tested, with the release enzyme being more pronounced at higher temperatures.

In conclusion, as with other proteinases of LAB (*4*, *9*, *11*), PrtL was detached from the cell envelope of *L. delbrueckii* subsp. *lactis* CRL 581. Although, the PrtL release was independent of the presence of $Ca^{2+}$ ions, it was related to the pH and temperature, being more pronounced at pH above 6.0 and at high temperature. PrtL was stable for 24 h at 4 °C; its stability was lower at acidic pH and was affected at temperatures over 25 °C, in particular, the released form of the enzyme.

Cheese is essentially a microbial fermentation of milk by selected LAB, whose main role is to produce lactic acid from lactose, which causes the pH of the curd to decrease. The final pH after cheese manufacture ranges from 4.6 to 5.6, depending upon the buffering capacity of the curd (*16*). Under the pH, temperature, and fermentation time conditions found during the manufacture of these fermented milk products, PrtL would remain active either bound to the cell or free in the supernatant. Thus, proteolysis by *L. delbrueckii* subsp. *lactis* CRL 581 could occur throughout the ripening period to contribute to the development of the organoleptic properties and beneficial health effects of cheeses.

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