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Modulation of the Cell-Surface Proteinase Activity of Thermophilic Lactobacilli by the Peptide Supply

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Abstract. The proteolytic system of thermophilic lactobacilli is considered important for bacterial nutrition as well as for the formation of flavor and texture in fermented products. We investigated the influence of peptide content on the cell surface proteinase and intracellular aminopeptidase activities from seven thermophilic lactobacilli strains. The proteinase activities were remarkably reduced in cells grown in the peptide-rich medium MRS or in a chemically defined medium supplemented with Casitone compared with those found in a synthetic medium. The degree of inhibition observed was strain dependent. When proteinase activities were analyzed by their hydrolytic patterns of α- and β-casein degradation, four types of P_{III}-caseinolytic cleavage specificity were distinguished. *Lactobacillus helveticus* strains possessed aminopeptidase activities with broader specificity than those found in *L. delbrueckii* subsp. *lactis* strains. However, the aminopeptidase activities were not influenced by the peptide content of the medium.

The thermophilic lactobacilli are the main starter cultures used in industrial fermentations for hard cheese and yoghurt productions. The growth rate of these microorganisms in milk depends on the production of proteolytic and peptidolytic enzymes, which, together with the respective peptide and amino acid transport systems, coordinately act to use casein as an external source for essential amino acids [9]. Their proteolytic system not only is vital to bacterial nutrition, but also contributes to the formation of flavor and texture in fermented products [11, 17]. According to their substrate specificity, the serine proteinases of lactic acid bacteria are classified in two types, P_I and P_{III} [9]. The P_I-type proteinases degraded predominantly β-casein, while the P_{III}-type proteinases hydrolyzed both α- and β-casein. Most of the cell envelope-associated proteinases found in mesophilic lactobacilli are P_I-type enzymes. In contrast, most thermophilic lactobacilli proteinases hydrolyzed α- and β-casein (P_{III}-type), although P_I-type enzymes have also been described (i.e., the cell-bound proteinases found in *Lactobacillus delbrueckii* strain BGPF1 and *Lactobacillus acidophilus* strains CH2 and V74 [3]). Genetic studies suggested that the proteinases found in thermophilic lactobacilli may represent a novel type of proteinase and that the genes encoding them may differ from those described in lactococci [15].

Among the lactic acid bacteria, the proteolytic system of lactococci has received considerably more attention than that of lactobacilli. Early experiments showed that synthesis of the lactococcal cell wall proteinase is reduced during bacterial growth in rich media compared with milk media [12]. Recently, Guedon and coworkers [5] showed that the activities of at least three aminopeptidases (PepN, PepC, and PepX) and the expression of the oligopeptide transport system (Opp) were greater in lactococcal cells grown in a synthetic medium than in media containing complex peptides. The P_{III}-type serine proteinase activity of *Lactobacillus helveticus* CRL 1062 has also been found to be modulated according to the growth medium used to culture the bacteria [7]. The highest proteinase levels were found during cell growth in a simplified chemically defined medium (SCDM), while the synthesis of this enzyme was inhibited in SCDM supplemented with Casitone, casamino acids, or β-casein.

The aim of this work was to compare the proteinase

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activities of five strains of *L. helveticus* and two *L. delbrueckii* subsp. *lactis* strains after their growth in media with different peptide compositions. The effect of peptide supply on aminopeptidase activities of these thermophilic lactobacilli was also studied.

Materials and Methods

Microorganisms, media, and growth conditions. The following strains of thermophilic lactobacilli were used in this study: *L. delbrueckii* subsp. *lactis* (*L. lactis*) CRL 581 and CRL 654 and *L. helveticus* CRL 974 (ATCC 15009), CRL 1062, CRL 1176 (ATCC 15807), CRL 1177, and CRL 1178 were obtained from the Centro de Referencia para Lactobacilos (CERELA, Tucumán, Argentina). *L. helveticus* CRL 1176 showed a proteinase-negative phenotype and was used as a negative control [8].

Working cultures of lactobacilli were routinely grown in the peptide-rich medium MRS [1] at 40°C for 16 h. To eliminate carryover nutrients, the cells were harvested by centrifugation at 8000*g* for 15 min, washed twice in sterile 50 mM phosphate buffer (pH 7.0), and resuspended to the original volume. This cell suspension was used to inoculate the different media at an initial optical density at 560 nm (OD_{560}) of 0.05–0.08. The chemically defined medium (CDM) described previously [7] was supplemented with 10 mg/ml adenine, 10 mg/ml guanine, and 1 mg/ml cyanocobalamin. These compounds were necessary to allow growth of *L. lactis* strains. When indicated, CDM was supplemented with 1% Casitone (Difco Laboratories, Detroit, MI).

Cell-free extracts. Cells grown in the different media were harvested by centrifugation at the exponential growth phase (OD_{560} = 0.65), washed twice with saline supplemented with 10 mM $CaCl_2$, and resuspended to a final OD_{560} of approximately 10 in 100 mM phosphate buffer (pH 7.0). Cell-free extracts were prepared by adding glass beads (0.15–0.25 mm diameter, Sigma Chemical Co., St. Louis, MO) to the bacterial cell suspensions and by shaking for 7 min at 4°C in a vortex at maximum speed. Glass beads, cell debris, and unbroken cells were removed by centrifugation (10,000*g*, 10 min, 4°C).

Proteinase activity. The proteinase (PrtH) activity of whole cell suspensions was measured in 50 mM phosphate buffer, pH 7.0, at 40°C with the chromogenic substrate Succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide (S-Ala; Sigma) as described by Exterkate [2]. One unit of proteinase (UE) was defined as the amount required to liberate 1 nmol of nitroaniline per min. Specific activity was expressed as UE per mg protein. Cell lysis was determined by following the release of lactate dehydrogenase (LDH) by the method of Thomas [16].

Aminopeptidase activity. The aminopeptidase activity was measured at 40°C against the following substrates (20 mM): L-alanine-*p*-nitroanilide (Ala-*p*NA), L-leucine-*p*-nitroanilide (Leu-*p*NA), L-arginine-*p*-nitroanilide (Arg-*p*NA), L-methionine-*p*-nitroanilide (Met-*p*NA), and L-proline-*p*-nitroanilide (Pro-*p*NA) (Sigma). The reaction mixture contained 100 μl of substrate stock solution, 850 μl of 0.2 M Tris-HCl buffer (pH 7.0), and 50 μl of enzyme extract. The reaction was stopped by the addition of 0.5 ml of 80% acetic acid. The samples were centrifuged, and subsequently the released nitroaniline was spectrophotometrically followed at 410 nm. One unit of aminopeptidase activity was defined as the amount of enzyme that hydrolyzed 1 μmol of substrate per min.

Casein hydrolysis. Washed cells, harvested from the different media, were suspended in 100 mM phosphate buffer (pH 7.0) and allowed to utilize the residual intracellular amino acids for 30 min at 40°C. Casein degradation was carried out as described previously [6]. Washed whole cells (OD_{560} = 10) were incubated with 5 mg/ml of substrate, dissolved in 100 mM phosphate buffer (pH 7.0), at a ratio of 1:1 (vol/vol) at 40°C for 3 h. As substrates, α- and β-casein (Sigma) were used. After various time intervals, samples were centrifuged, and the supernatants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as described previously [10]. Either Coomassie Brilliant Blue R-250 or silver staining (Bio-Rad Laboratories, Richmond, CA) was used to visualize the proteins after SDS-PAGE.

Protein determination. Protein concentration was determined by use of a protein assay according to the manufacturer's instructions (Bio-Rad).

Results and Discussion

Proteinase activities of thermophilic lactobacilli. The cell envelope proteinases of seven thermophilic lactobacilli strains were investigated by their action on the chromophoric substrate S-Ala (Table 1), as well as by SDS-PAGE with α- and β-casein as substrates (Figs. 1, 2). Bacterial cells were grown in CDM, a basal medium that contains all amino acids necessary for bacterial growth; in MRS, a peptide-rich medium with a complex nitrogen source; and in CDM supplemented with Casitone. Addition of a pancreatic digest of casein (Casitone) to CDM supplies the basal medium mainly with small peptides. As shown in Table 1 and Figs. 1 and 2, the strains of *L. helveticus* and *L. lactis* analyzed displayed different proteolytic activity levels, depending upon the growth medium. In control experiments, it was demonstrated that Casitone did not show any direct inhibitory effect on the proteolytic activity of these strains.

With the exception of *L. helveticus* CRL 1176, the proteinase activities of cells grown in CDM medium were higher than those grown in MRS or CDM supplemented with 1% Casitone (Table 1). The highest specific proteinase activity was detected in *L. helveticus* CRL 1177 and *L. lactis* CRL 581 cultured in CDM. An approximately 100-fold reduction in enzyme activity from these strains was observed in whole cells grown in peptide-rich media. However, although peptides substantially affected the cell envelope proteinase activities of thermophilic lactobacilli, this effect was strain dependent, and inhibitory factors lower than 41-fold were observed for the other strains.

The kinetics of α- and β-casein hydrolysis by whole cells grown in CDM and CDM supplemented with Casitone are shown in Figs. 1 and 2, respectively. The strains grown in CDM medium degraded α- and β-casein faster than did those grown in CDM plus Casitone. Thus, the proteinases of the investigated thermophilic lactobacilli have a caseinolytic activity comparable to that of the P_{III}-type of lactococcal proteinase.

The hydrolytic patterns of α- and β-casein degrada-

Table 1. Proteinase activities of thermophilic lactobacilli after their growth on a chemically defined medium (CDM), CDM supplemented with Casitone, and MRS

Strains	Specific proteinase activity[a,b]				
	CDM	CDM plus Casitone	Inhibition factor[c]	MRS	Inhibition factor[d]
L. lactis CRL 581	829 ± 30	8 ± 0.3	104	6 ± 0.2	138
L. lactis CRL 654	244 ± 10	9 ± 0.4	27	6 ± 0.3	41
L. helveticus CRL 974	129 ± 5	42 ± 2	3	6 ± 0.2	22
L. helveticus CRL 1062	192 ± 8	15 ± 0.8	13	6 ± 0.2	32
L. helveticus CRL 1176	14 ± 0.8	14 ± 0.9	1	4 ± 0.1	3
L. helveticus CRL 1177	580 ± 22	6 ± 0.2	97	5 ± 0.2	116
L. helveticus CRL 1178	140 ± 6	12 ± 0.5	12	5 ± 0.2	28

[a] Values are the means from three independent experiments ± SD.
[b] Specific activity is expressed as nmol min^{-1} mg $protein^{-1}$. The lactate dehydrogenase activity was less than 0.06% of the total activity in each cell extract.
[c] Ratio between the values obtained in CDM and CDM plus 1% Casitone.
[d] Ratio between the values obtained in CDM and MRS.

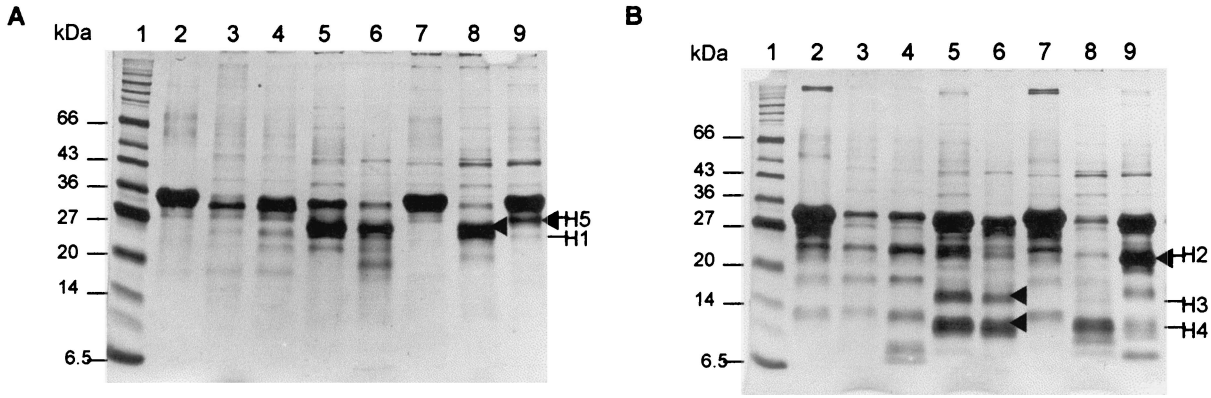

Fig. 1. SDS-PAGE analysis of α-casein (A) and β-casein (B) hydrolysis by thermophilic lactobacilli grown on CDM medium. Lane 1, molecular mass markers; lane 2, starting substrate (control); lane 3, *L. lactis* CRL 581; lane 4, *L. lactis* CRL 654; lane 5, *L. helveticus* CRL 974; lane 6, *L. helveticus* CRL 1062; lane 7, *L. helveticus* CRL 1176; lane 8, *L. helveticus* CRL 1177; and lane 9, *L. helveticus* CRL 1178. The peptide band positions are indicated by arrows.

tion observed for both strains of *L. lactis* were similar (Figs. 1, 2). The cleavage of α-casein by cells grown in CDM with Casitone released a main peptide with a relative molecular mass (M_r) close to 25 kDa (Fig. 2A, band L1) and four minor peptides bands with an Mr lower than 14 kDa (Fig. 2A). The β-casein electrophoretograms were characterized for two strong peptide bands (about 24 and 12 kDa, respectively; Fig. 2B, bands L2–L3), and two fainter bands of M_r lower than 14 kDa (Fig. 2B). The CDM-grown cells hydrolyzed α- and β-casein into smaller peptides (Fig. 1A, B), the rate of hydrolysis by CRL 581 strain being higher than that for CRL 654.

On the other hand, the degradation patterns of α- and β-casein by *L. helveticus* was strain dependent. Strains CRL 974, CRL 1062, and CRL 1177 displayed an electrophoretic profile on α-casein characterized by a strong peptide band with an approximate M_r of 25 kDa (Fig. 1A, band H1). Furthermore, the CRL 974 and CRL 1062 β-casein hydrolytic patterns were similar. These electrophoretograms were characterized by three main peptides (between 23 and 6.5 kDa; Fig. 1B, bands H2–H4), while the CRL 1177 proteinase degraded β-casein into smaller peptides. *L. helveticus* CRL 1178 grown on CDM medium showed a reduced rate of α- and β-casein hydrolysis with major peptide products of 27 kDa (H5) and 23 kDa (H2), respectively. On the contrary, this strain on CDM supplemented with Casitone (Fig. 2; see also Table 1) or in MRS (Table 1) showed a proteinase-negative phenotype. As expected, no detectable degradation of α- and β-casein was observed with *L. helveticus* CRL 1176.

Taken together, these data suggest that the proteinases found in the studied thermophilic lactobacilli would

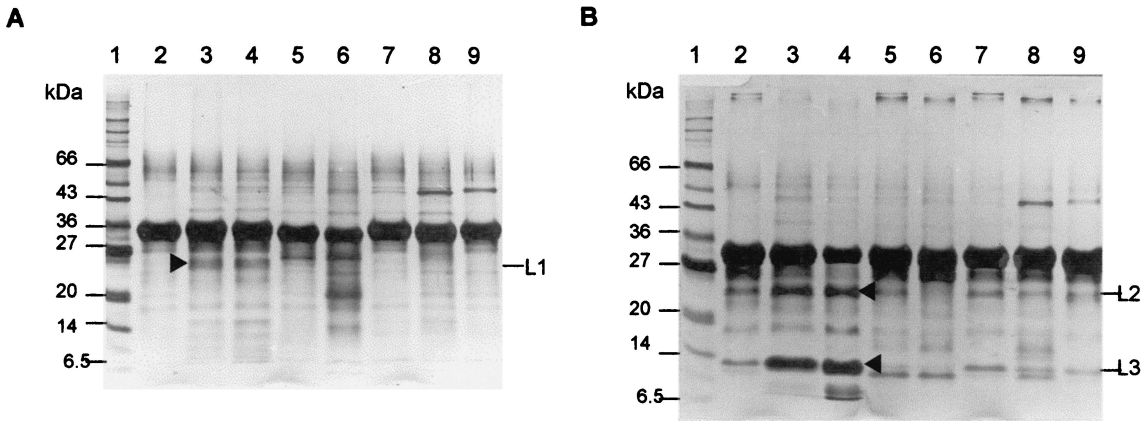

Fig. 2. SDS-PAGE analysis of α-casein (A) and β-casein (B) hydrolysis by thermophilic lactobacilli grown on CDM supplemented with 1% Casitone. Lane 1, molecular mass markers; lane 2, starting substrate (control); lane 3, *L. lactis* CRL 581; lane 4, *L. lactis* CRL 654; lane 5, *L. helveticus* CRL 974; lane 6, *L. helveticus* CRL 1062; lane 7, *L. helveticus* CRL 1176; lane 8, *L. helveticus* CRL 1177; and lane 9, *L. helveticus* CRL 1178. The peptide band positions are indicated by arrows.

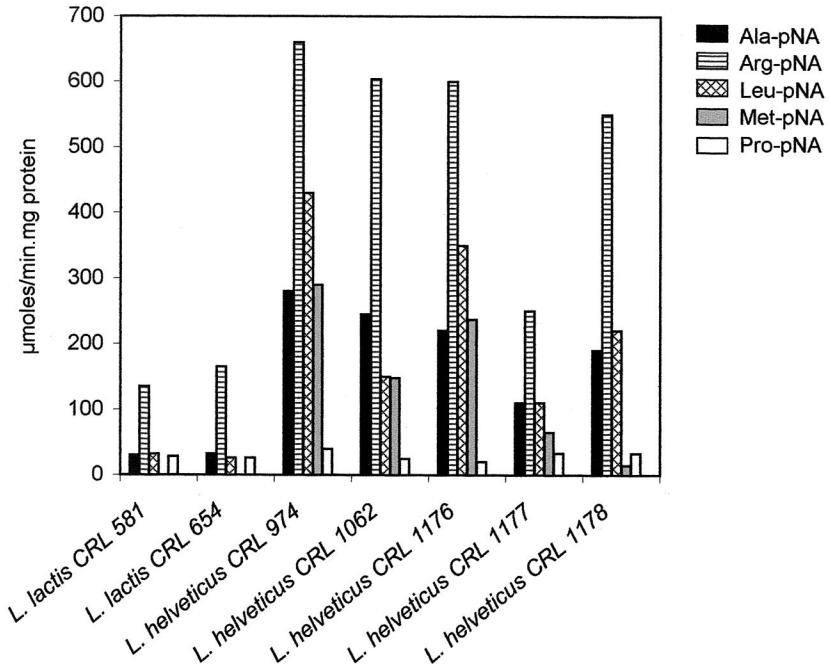

Fig. 3. Aminopeptidase activities of thermophilic lactobacilli strains grown on MRS medium towards different chromogenic substrates.

differ from each other or that probably the effect exerted by repressing factor(s) present in Casitone and MRS on proteinase activity would be mediated by different mechanisms of proteinase regulation.

Peptidase activities of thermophilic lactobacilli. Cell-free extracts of thermophilic lactobacilli, grown in the peptide-rich MRS medium, were tested for peptidase activities with five chromogenic substrates. As shown in Fig. 3, the highest levels as well as the wider spectrum of aminopeptidase activities were detected in the *L. helveticus* strains. *L. helveticus* CRL 974 was the most peptidolytic microorganism, being active against all the substrates used in this study. On the contrary, *L. lactis* strains were not able to hydrolyze Met-*p*NA. All strains analyzed showed the highest aminopeptidase activities on Arg-*p*NA, while lower activity was detected towards the substrate Pro-*p*NA. However, the *L. helveticus* strains analyzed displayed higher proline aminopeptidase activities than those described in the literature for lactococci or *L. casei* (18- and 4.5-fold higher, respectively [14]).

Activity levels of these enzymes were also deter-

mined in *L. lactis* CRL 581 and CRL 654 and in *L. helveticus* CRL 974 and CRL 1062 grown in CDM. Unlike the proteolytic enzymes, similar aminopeptidase activities were observed in both media for the strains tested (data not shown). It can be concluded that the peptide supply showed no inhibitory effect on the peptidase activity in the investigated strains. These results are in agreement with those found by Gilbert et al. [4] and Meijer et al. [13] for Pep N activities of *L. helveticus* NCDO 766 and *Lactococcus lactis* subsp. *cremoris* SK1128, respectively.

The presence of a cell envelope proteinase and peptidases in thermophilic lactobacilli is important for their use as hard cheese starters to liberate desirable flavor peptides as well as to prevent bitterness in ripened cheeses. Swiss cheeses are especially rich in free proline, which is associated with the sweet flavor. Therefore, the amount of free proline will depend directly on the activity of the thermophilic lactobacilli peptidases. A better characterization of the proteolytic system found in *L. helveticus* and *L. delbrueckii* subsp. *lactis* will contribute to providing a strong foundation to improve the choice of starters and for a better control of the fermentation processes.

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