Proteolytic Activity of *Lactobacillus casei* Subsp. *casei* IFPL 731 in a Model Cheese System

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The role played by the cell-envelope proteinase and the aminopeptidase activity of *Lactobacillus casei* subsp. *casei* IFPL 731 isolated from goat's milk cheese has been dilucidated, adding the cell wall extract and the intracellular fraction of Prt$^-$ and Amp$^-$ variants to cheese slurries, in which residual rennet was inactivated. Primary proteolysis of casein was mainly conducted by rennet, but once this was inactivated, the main responsibility of casein hydrolysis was cell-envelope proteinase activity, followed by plasmin and microbial intracellular proteinases. Formation of the amino acid nitrogen fraction was explained to a great extent by the action of the intracellular aminopeptidase activity, but it was demonstrated that the cell-envelope proteinase plays an important role as a rate-limiting factor for this nitrogen formation. Hydrophobic peptides were hydrolyzed during incubation, and Leu and Lys amino acid residues were produced, as a consequence of the peptidase activity present in the slurries.

Keywords: *Lactobacillus casei, proteolysis, model cheese system*

INTRODUCTION

Casein hydrolysis during cheese ripening is mainly due to the action of residual rennet on α_{s1}-casein and subsequently followed by proteolysis produced by proteinases and peptidases of starter lactic acid bacteria (Kunji *et al.*, 1996). During cheese ripening, nonstarter lactic acid bacteria proliferate, often reaching densities of approximately 10^7 cfu g^{-1} cheese, for extended periods of time. These bacteria can actively contribute to proteolysis and flavor development (Peterson and Marshall, 1990) and have been successfully used as adjunct starters to shorten the ripening time and enhance the characteristic flavor of the cheese (El Abboudi *et al.*, 1992a,b; Lynch *et al.*, 1996).

The proteolytic activity of lactic acid bacteria has been traditionally assessed using as substrates skim milk and sodium caseinate solutions, and more recently chromophoric substrates (Law and Mulholland, 1995). Nevertheless, cheese is a very complex system, and results obtained with liquid substrates cannot be straightforwardly extrapolated to cheese. Therefore, some model systems consisting of aseptic cheeses or cheese slurries have been developed in an attempt to reproduce real cheese conditions (Farkye *et al.*, 1995). These slurry systems have been used in our laboratory to assess the proteolysis produced by both lactococci (Rodríguez *et al.*, 1996) and mesophilic lactobacilli (Parra *et al.*, 1996), added to the milk as adjunct starters.

Previously, we have demonstrated that the use of the adjunct *Lactobacillus casei* subsp. *casei* IFPL 731 favors the development of the organoleptic characteristics of goat's milk cheese (Requena *et al.*, 1992). The cell-envelope proteinase of *L. casei* IFPL 731 has been purified and characterized (Fernández de Palencia *et al.*, 1995, 1997a) as well as some components of its aminopeptidase system (Fernández de Palencia, 1996).

Later, we have reported the isolation and characterization of some proteolytic system-deficient mutants of this bacterium (Fernández de Palencia *et al.*, 1997b).

To elucidate the role of some components of the proteolytic system of *L. casei* IFPL 731, we have studied in the present work the proteolysis produced by the cell wall and intracellular fraction of some proteinase- and aminopeptidase-deficient mutants, using a cheese model system.

MATERIALS AND METHODS

Microorganism and Culture Conditions. *Lactobacillus casei* subsp. *casei* IFPL 731 and its proteinase-deficient IFPL 732 (Prt$^-$) variant, and aminopeptidase-deficient IFPL 733 (Amp$^-$) variant, obtained as described before (Fernández de Palencia *et al.*, 1997b), were maintained as frozen stock culture at −80 °C in MRS broth (Oxoid Co., Basingstoke, England), containing 25% glycerol.

L. casei IFPL 731 strain and its variants were subcultured twice overnight in MRS broth at 30 °C, before being used to inoculate (1% inoculum) 4 L of reconstituted skim milk supplemented with 0.002% manganese sulfate and 0.004% sodium formiate. Growth of the Prt$^-$ variant was supplemented with 0.2% caseiton (Difco, Detroit, MI). Cultures were grown at 30 °C until they reached a final pH of 5.2 (approximately 10^9 cfu mL^{-1}) and were subsequently harvested as described previously (Fernández de Palencia *et al.*, 1995).

Enzymatic Fractions. *Cell Wall Extract.* Cell wall extracts (CWE) of *L. casei* IFPL 731 and its variants 732 (Prt$^-$) and 733 (Amp$^-$) were obtained as described by Fernández de Palencia *et al.*, (1995), modifying the added concentrations of lysozyme (Boehringer Manheim, Manheim, Germany) and mutanolysin (10 000 units; Sigma Chemical Co., St. Louis, MO) (1 mg mL^{-1} and 25 μL, respectively). After incubation at 37 °C for 3 h, the solubilized material, CWE, was collected by centrifugation (17000*g*, 30 min). Solid ammonium sulfate was added to the CWE to a final concentration of 14% w/v. Insoluble material was removed by centrifugation (20000*g*, 20 min, 4 °C), and additional ammonium sulfate was added to a final concentration of 35% w/v to precipitate the material

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containing the proteinase. After subsequent centrifugation under the same conditions described above, the pellets were disolved in 50 mM Tris-HCl buffer, pH 7.0.

Intracellular Fraction. Sediments of protoplasts, obtained after centrifugation of the cells incubated with lysozyme and mutanolysin, were disrupted in 20 mM Tris-HCl buffer, pH 7.5, using glass beads (Sigma Chemical Co.) as described previously (Fernández-Esplá *et al.*, 1997). Then the clear supernatant designated as intracellular fraction (IC) was sterilized trough 0.22 μm filters (Millipore Co., Bedford, MA). The fractions CWE and IC were stored at −80 °C until further use.

Preparation of Cheese Slurries. Cheese slurries were prepared from commercial pasteurized skim cow's milk, as described by Rodríguez *et al.* (1996). To each 100 g of the slurry was added 40 mL of 4% sterile NaCl. To enhance the primary proteolysis of casein, 0.0015% animal rennet was added to the slurries under aseptic conditions, followed by incubation at 30 °C for 48 h; 0.02% sodium azide was added to the slurries to prevent microbial growth. To inactivate the rennet, the slurries were further heated at 70 °C for 15 min and were subsequently stored at 4 °C, until the next day.

Approximately 140 g of cheese slurries was mixed in a orbital shaker (Certomat, H, Braun, Germany) for 15 min with 10 mL of CWE extracts of *L. casei* IFPL 731 (slurry A) and its variants 732 (Prt^-) (slurry B) and 733 (Amp^-) (slurry C). After incubation at 30 °C for 2 days, 10 mL of the correspondent IC was added to the slurries, and incubation was prolonged for 13 days at the same temperature. Experiments were run in duplicate, and samples were analyzed at 0, 2, 7, and 15 days of incubation.

Enzymatic Activity. One gram of cheese slurries was mixed with 1 mL of 50 mM sodium phosphate buffer, pH 7.0, shaken in a vortex for 2 min at room temperature, and centrifuged at 10000*g*, 10 min, 4 °C. Supernatants were used for determination of enzyme activity.

Proteinase activity was measured in 50 mM sodium phosphate buffer, pH 7.0 at 30 °C, by following the hydrolysis of the substrate MeOsuc-Arg-Pro-Tyr-*p*NA (MS-Arg) (Kabi Diagnostica, Sweden) as described previously (Martín-Hernández *et al.*, 1994). Aminopeptidase activity was measured in sodium phosphate buffer at 37 °C, by following the hydrolysis of the substrate Leu-*p*-nitroanilide (Leu-*p*NA) (Sigma Chemical Co.) by the method described by Exterkate (1984). One unit of enzymatic activity was defined as the amount of enzyme required to release 1 nmol of *p*-nitroaniline under assay conditions. The molar extinction coefficient of *p*-nitroaniline (E_{410}) was 8800 M^{-1} cm^{-1}.

Proteolysis. Samples were analyzed for total nitrogen (TN) by the Kjeldahl method (Official Methods of Analysis, 1975). Non-casein nitrogen (NCN) and non-protein nitrogen (NPN) were determined as described by Kuchroo and Fox (1982). Amino acid nitrogen (AN) was determined using the trinitrobenzenesulfonic acid (TNBS) reaction, as described by Kuchroo *et al.* (1983).

Peptide Analysis by HPLC. Fifty microliters of the soluble non-casein nitrogen filtered through a Durapore 0.45 μm filter (Millipore Co.) was analyzed by reverse-phase HPLC. A Beckman System Gold HPLC and a System Gold Software data acquisition system (Beckman Instruments Inc., S. Ramón) were used.

Separations were performed at 40 °C, following the method described by González de Llano *et al.* (1995), using a C-18 nucleosil column (5 μm, 300 Å, 250 × 4.6 mm) (Macherey Nagel, Düren, Germany). Solvents A and B were 0.1% aqueous trifluoroacetic acid (TFA; Merck Darmstadt, Germany) and 0.1% TFA in acetonitrile/water (60:40 v/v), respectively. A flow rate of 1 mL min^{-1} was employed with detection at 220 nm.

The aromatic amino acids tyrosine, phenylalanine, and trytophan were run on the HPLC to identify their retention times. Likewise, the α_{s1}-casein (1−23) fragment [α_{s1}-CN(f1−23)] and its hydrolysis products, the peptides fragment 1−9 and fragment 10−23 obtained after incubation with *L. casei* 731 IFPL cell wall proteinase, were analyzed in 50 mM imidazole buffer, pH 6.5, as described previously (Martín-Hernández *et al.*, 1994), to identify them in the cheese slurries on the basis of their retention times.

Table 1. Evolution of Non-Casein Nitrogen (NCN), Non-Protein Nitrogen (NPN), and Amino Acid Nitrogen (AN) Expressed as Percent of Total Nitrogen (TN) during Incubation of Cheese Slurries at 30 °C

cheese slurry[a]	time (days)	NCN (% TN)[b]	NPN (% TN)[b]	AN (% TN)[c]
control	0	$2.10^a \pm 0.03^d$	$1.85^a \pm 0.10$	$0.01^a \pm 0.001$
	2	$2.30^b \pm 0.03$	$1.85^c \pm 0.23$	$0.01^b \pm 0.004$
	7	$2.31^c \pm 0.05$	$1.59^c \pm 0.21$	$0.01^c \pm 0.001$
	15	$4.04^c \pm 0.07$	$2.27^c \pm 0.32$	$0.01^d \pm 0.006$
A	0	$2.18^a \pm 0.03$	$1.88^a \pm 0.10$	$0.01^a \pm 0.001$
	2	$3.58^a \pm 0.07$	$2.96^a \pm 0.06$	$0.10^a \pm 0.030$
	7	$4.27^a \pm 0.21$	$2.89^a \pm 0.34$	$0.35^a \pm 0.060$
	15	$8.08^a \pm 0.58$	$4.63^a \pm 0.23$	$0.89^a \pm 0.010$
B	0	$2.15^a \pm 0.03$	$1.93^a \pm 0.10$	$0.01^a \pm 0.001$
	2	$3.31^a \pm 0.19$	$1.37^b \pm 0.11$	$0.06^a \pm 0.030$
	7	$2.97^b \pm 0.58$	$2.39^b \pm 0.33$	$0.24^b \pm 0.020$
	15	$6.4^b \pm 0.26$	$3.35^b \pm 0.40$	$0.53^b \pm 0.070$
C	0	$2.13^a \pm 0.03$	$1.79^a \pm 0.10$	$0.01^a \pm 0.001$
	2	$3.64^a \pm 0.17$	$2.03^c \pm 0.24$	$0.07^a \pm 0.009$
	7	$3.98^a \pm 0.42$	$2.93^a \pm 0.16$	$0.22^b \pm 0.110$
	15	$8.70^a \pm 0.57$	$4.26^a \pm 0.48$	$0.80^c \pm 0.050$

[a] A: slurry added with CWE and IC of *L. casei* 731. B: slurry added with CWE and IC of 732 (Prt^-). C: slurry added with CWE and IC of 733 (Amp^-). [b] Mean ± standard error of duplicate determination on two replicates. [c] Mean ± standard error of triplicate determination on two replicates. [d] Different letters in the same column indicate significative differences ($p < 0.05$) between the cheese slurries at the same incubation time.

Free Amino Acids. Free amino acid analysis was performed as described by Ventas *et al.* (1992) in the 3% sulfosalicylic acid soluble fraction (Mondino *et al.*, 1972) using a Biochrom 20 amino acid analyzer (Pharmacia LKB, Uppsala, Sweden).

Statistical Analysis. Statistical study of results was performed using one-way analysis of variance to determine significant differences ($p < 0.05$) in proteolysis, among slurries prepared with enzymatic fractions of *L. casei* IFPL 731 and its Prt^- and Amp^- variants.

RESULTS AND DISCUSSION

Enzymatic Activity in Cheese Slurries. Recovery of the proteinase and aminopeptidase activities in the slurries was about 15−20% (results not shown). In cheese slurries A and C, proteinase activity was lost up to 70%, after 7 days incubation. This is attributed to the high instability of the released cell wall proteinase from *L. casei* IFPL 731, which has been previously demonstrated (Fernández de Palencia *et al.*, 1997a). Broome *et al.* (1991) have also reported a decrease in the free proteinase activity present in cheddar cheese, during 48 weeks of ripening. On the other hand, aminopeptidase activity showed higher stability than the proteinase in the cheese slurries, and it was recovered up to 50%, after 15 days incubation. The level of aminopeptidase activity in cheese slurry C was very low over the incubation period.

Proteolysis. Evolution of proteolysis in the slurries was monitored via the ratios between the non-casein nitrogen (NCN), non-protein nitrogen (NPN), and amino acid nitrogen (AN), referred to total nitrogen (TN). The results of proteolysis are shown in Table 1.

The NCN/TN values after 2 days incubation of the control cheese slurries can be attributed to the action of rennet added during milk coagulation. In addition, rennet was added to enhance proteolysis in a later stage of the slurries preparation. With the inactivation of the rennet by heating the slurries at 70 °C for 15 min, the

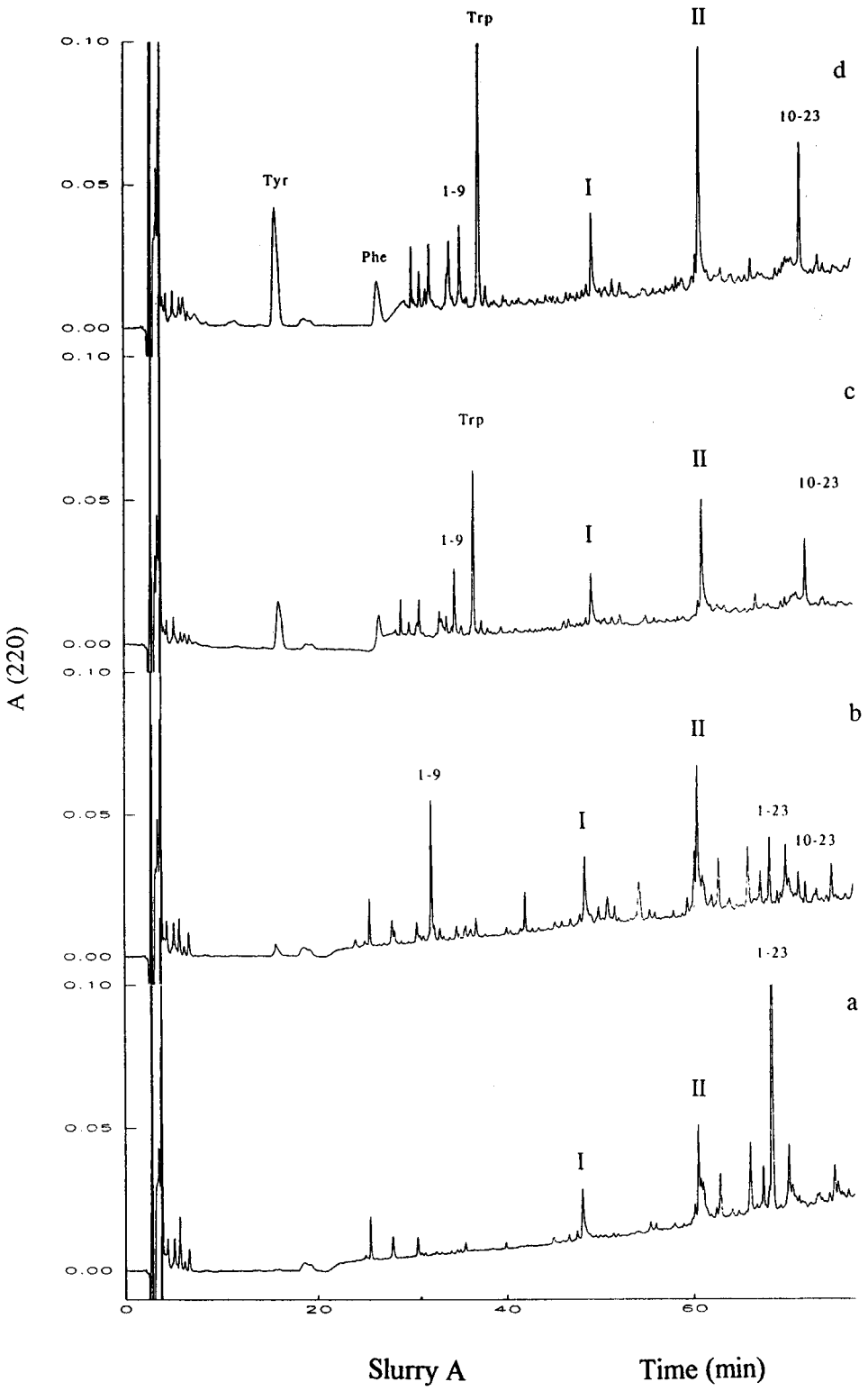

Figure 1. Reverse-phase HPLC pattern of soluble products contained in the non-casein nitrogen (NCN) fraction of cheese slurry A at 0 (a), 2 (b), 4 (c), and 7 (d) days of incubation at 30 °C.

increase in the NCN during 2 weeks incubation of the control slurries must have been the result of the casein hydrolysis produced by the milk plasmin. Besides the action of milk plasmin, the significant increase ($p < 0.05$) in the NCN of slurries A and C after 2 days incubation is mainly attributed to the proteinase activity added to the slurries with the microbial CWE's, which is absent in slurry B. From 7 days on, such an increase can also be explained by the presence of intracellular proteinases, active in casein hydrolysis. Such proteinases have been described in *Lactococcus* (Stepaniak *et al.,* 1996).

The crucial role of the residual rennet in α_{s1}-casein hydrolysis has been demonstrated on several occasions (Fox, 1989), this casein being the main substrate for proteolysis during cheddar cheese ripening (Creamer, 1975). Besides, the role of the starter proteinases on NCN formation in cheese has been reported by Exter-

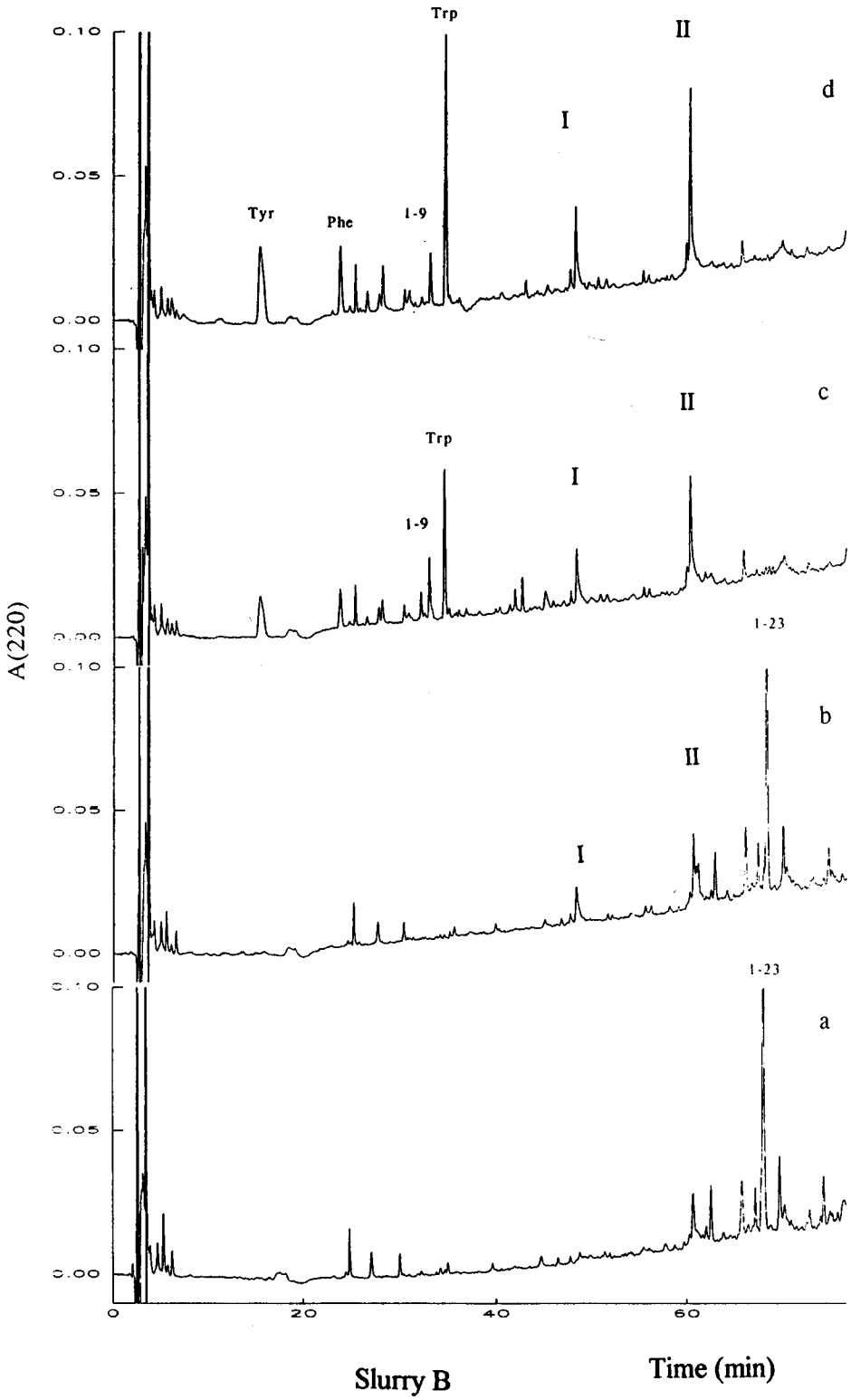

Figure 2. Reverse-phase HPLC pattern of soluble products contained in the non-casein nitrogen (NCN) fraction of cheese slurry B at 0 (a), 2 (b), 4 (c), and 7 (d) days of incubation at 30 °C.

kate and Alting (1995). The hydrolysis of the β-casein in Gouda cheese has been attributed to both the action of milk plasmin and the proteinase activity of the starter (Visser, 1993).

As for NCN/TN, levels of NPN/TN arose in all systems, mostly at the outset of the incubation period. Slurry B with no added proteinase activity showed the lowest levels of NPN after 15 days incubation. The values of this nitrogen component found in slurry B could be explained by the action of intracellular proteinases contained in the added IC's. The role of the lactococcal cell-envelope proteinase in the formation of the non-protein nitrogen during cheese ripening has been previously demonstrated using *Lactococcus* (Prt^-) in Cheddar cheese manufacture (Broome *et al.*, 1991).

Evolution of the AN/TN values during the incubation of the slurries was straightforwardly related to the addition of aminopeptidase activity with the microbial IC's. The amino acid nitrogen component, absent in the control slurry, increased at 7 and 15 days incubation in

slurry A, differing significantly ($p < 0.05$) from the levels found in slurry C, which contained the IC of the deficient strain 733 (Amp^-), and slurry B, which contained the IC of the parental strain. In fact, slurry B containing the total aminopeptidase activity showed the lowest ($p < 0.05$) AN content among all three slurries. The absence of the proteinase activity in slurry B would not furnish enough substrates for subsequent hydrolysis by peptidases. Therefore, the proteinase activity represents a limitation factor in the rate of formation of amino acids.

The role of the starter cell-envelope proteinase as a rate-limiting proteolysis factor has been reported by other authors (Farkye *et al.*, 1990; Law *et al.*, 1993). Nevertheless, the use of lactococci overexpressing proteinase activity as a starter for cheese manufacture has not led to an increase in secondary proteolysis, since peptidase activity is considered the main agent responsible for the formation of the AN/TN nitrogen fraction in cheese (Law *et al.*, 1993; Law and Mulholland, 1995).

Free Amino Acids. The amino acid values showed the same general trend as observed for amino acid nitrogen values, with the highest formation rate in slurry A and the lowest in slurry B. Before incubation of the slurries, the highest relative percentage of amino acids was found for the residues Glu (21%) and Gly (16%), eventually produced by the action of rennet. At the end of the incubation period, the total content of amino acids Leu and Lys was the highest with relative percentages of 11 and 12%, respectively. Substrates containing these two amino acid residues have been found to be preferably hydrolyzed by the intracellular enzymatic fraction of *L. casei* IFPL 731 (Requena *et al.*, 1993).

Besides, the deficiency in aminopeptidase activity of slurry C did not alter significantly the profile of the free amino acids, still being the residues Leu and Lys, the main components of this fraction.

HPLC Analysis of NCN Fractions. Figures 1 and 2 show the reverse-phase HPLC separation of peptides contained in the NCN fraction of slurries A and B during several days of incubation. The HPLC pattern of slurry C was very similar to that of slurry A (not shown).

At zero days incubation, a complex pattern of soluble peptides mostly produced by the action of rennet was obtained in all three slurries. At this moment, the main component of this peptide profile was fragment 1−23 derived from α_{s1}-casein.

The total amount of peptide material increased during incubation, but a clear distinct profile was observed between slurries. At 2 days incubation, the action of the cell-envelope proteinase added to slurries A and C containing the CWE of the parental strain resulted in the cleavage of fragment 1−23 and the formation of its hydrolysis products, peptides 1−9 and 10−23. This hydrolysis specificity is characteristic of the cell-envelope proteinase from *L. casei* IFPL 731, as has been demonstrated previously (Fernández de Palencia *et al.*, 1997a). Besides, as a consequence of the proteinase action, some other peptides were observed. On the contrary, slurry B did not show any cleavage of fragment 1−23 at 2 days incubation, due to its deficiency in proteinase activity.

Further cleavage of fragment 1−23 and other peptides took place in all three slurries, being completed at 4 days incubation and therefore attributed to the action of the added intracellular material. As a consequence, large or hydrophobic peptides exhibiting higher retention times on the reverse column were hydrolyzed to smaller peptides (less than 40 min retention time). The hydrolysis of hydrophobic peptides by the peptidase system of *L. casei* IFPL 731 suggests the debittering potential activity of this strain.

At the end of the incubation period, some hydrophobic peptides resistant to hydrolysis were still present in the chromatograms, two of them being products of casein hydrolysis by the rennet (peptides I and II) and the third (peptide 10−23), absent in slurry B, produced by the action of the cell-envelope proteinase. Peptide 1−9, present in the hydrophilic zone, was also resistant to further hydrolysis.

From the results described above, it can be concluded that in a model system consisting of cheese slurries with inactivated rennet, the proteinase of *L. casei* IFPL 731 plays an essential role not only in the primary hydrolysis of casein but also in amino acid nitrogen formation, being a rate-limiting factor for this proteolysis step. Besides, the aminopeptidase activity is the main agent responsible for amino acid nitrogen formation, releasing Leu and Lys as characteristic amino acid residues. The peptide profile of slurries in which hydrophobic fragments are progressively hydrolyzed suggests a potential debittering role of the peptidase system of this strain. The aminopeptidase activity deficiency, although it affected the total content of amino acid nitrogen, did not modify the final proportions of the different free amino acids.

Since these results were reported in a simple cheese model, we are currently scaling up these experiments to the manufacture of cheese.

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