Purification and Characterization of the Cell Wall Proteinase of *Lactobacillus casei* Subsp. *casei* IFPL 731 Isolated from Raw Goat's Milk Cheese

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A cell wall proteinase from *Lactobacillus casei* subsp. *casei* IFPL 731 has been released in the presence of ethylenediaminetetraacetic acid and purified by ammonium sulfate precipitation, hydrophobic interaction, and ion exchange chromatography. Its molecular mass estimated by SDS−PAGE was about 150 kDa. Maximum activity was reached at pH 6.0 and 40 °C. Activity was completely inhibited by phenylmethanesulfonyl fluoride, suggesting that the enzyme belongs to the serine group of proteinases. It was very unstable even at 4 °C, and the Ca^{2+} ion was ineffective as a stabilizing agent. It preferentially hydrolyzed β-casein and clove mainly bond 9−10 of the α_{s1}-casein(1−23) peptide. On the basis of its specificity toward caseins, an α_{s1}-casein(1−23) fragment, and two different charged chromophoric peptides, the proteinase can be classified as a mixed-type variant different from those identified with lactococcal proteinases.

Keywords: *Lactobacilli; proteinase; characterization; specificity*

INTRODUCTION

Lactic acid bacteria are essential microorganisms in the production of fermented dairy products. Besides lactococci, commonly used in starter cultures, research on selection and characterization of *Lactobacillus* strains has increased over the past 10 years in response to their growing importance. These nonstarter lactic acid bacteria colonize cheese during the late stages of ripening, eventually reaching high densities and contributing to proteolysis and flavor development (Peterson and Marshall, 1990). In addition, they have the capacity to inhibit undesirable nonstarter lactic acid bacteria present as contaminants in cheese (Khalid and Marth, 1990; Martley and Crow, 1993).

Lactic acid bacteria possess a large serine proteinase located in the cell envelope (CEP), which is involved in the degradation of caseins and large peptides derived from casein by the action of rennet. The CEPs of a number of *Lactococcus lactis* strains (Lc-CEP) have been characterized biochemically and genetically and show significant sequence homology with the family of subtilisin-like serine proteinases [see review by Kunji *et al.* (1996)]. Two major proteinase types (P_I and P_{III}) have been distinguished according to their cleavage specificity with respect to casein (Visser *et al.*, 1986). More recently, a study was made of a number of lactococci CEP variants intermediate between the P_I and P_{III} types, classified on the basis of their specificity toward the α_{s1}-casein(1−23) fragment [α_{s1}-CN(f1−23)] and two different charged chromophoric peptides (Exterkate *et al.*, 1993).

CEPs in lactobacilli (Lb-CEP) have been described [see review by Kok and de Vos (1994)]. Purification and characterization of Lb-CEP have been reported for *Lb. delbrueckii* subsp. *bulgaricus* (Laloi *et al.*, 1991) and *Lb. helveticus* (Yamamoto *et al.*, 1993; Martín-Hernández *et al.*, 1994). The cell-wall-bound proteinase of *Lb. paracasei* subsp. *paracasei* NCDO 151 (formerly *Lactobacillus casei*) has been purified (Naes and Nissen-Meyer, 1992). Although a number of variants of the enzyme in species of lactobacilli have been suggested (Kojic *et al.*, 1995), the hybridization and restriction maps of the chromosomally located proteinase gene region of *Lb. paracasei* subsp. *paracasei* NCDO 151 and *L. casei* HN14 indicate high similarity to the proteinase of *Lc. lactis* subsp. *cremoris* Wg2 (Holck and Naes, 1992; Kojic *et al.*, 1991).

In an earlier paper (Fernández de Palencia *et al.*, 1995) we studied the release of the cell envelope proteinase of *Lb. casei* subsp. *casei* IFPL 731, a microorganism that had been isolated from raw goat's milk cheese and successfully used as an adjunct starter for this type of cheese (Requena *et al.*, 1992). In the present work we describe the purification, characterization, and specificity of the enzyme toward α_{s1}- and β-casein, fragment 1−23 of α_{s1}-casein [α_{s1}-CN (f1−23)], and two chromophoric peptides for comparison with the diversity of Lc-CEP variants reported by Exterkate *et al.* (1993).

MATERIALS AND METHODS

Microorganism and Growth. *Lb. casei* subsp. *casei* IFPL 731 was maintained as frozen stock culture at −80 °C in MRS broth (Oxoid Co., Basingstoke, England) containing 25% glycerol. The microorganism was grown in 10% enriched skim milk (Fernández de Palencia *et al.*, 1995) at 30 °C. Bacteria were subcultured twice overnight, before inoculation of the batch culture at about 10^7 cfu mL^{-1} (final concentration). Bacterial growth was estimated by plating out on MRS agar and counting after 3 days of incubation at 30 °C.

Enzyme Purification. *Release of CEP.* Cells from a 10 L culture (overnight at 30 °C, final pH 5−5.2) were harvested and washed as described previously (Fernández de Palencia *et al.*, 1995). CEP was released according to the method of Naes and Nissen-Meyer (1992), by incubating the cell pellet at 25 °C for 1 h in 20 mM Bis-Tris buffer, pH 6.0, containing 10 mM ethylenediaminetetraacetic acid (EDTA) (20 mL of buffer/L of culture). After centrifugation (17500*g*, 4 °C, 30 min), the supernatant fluids were collected. This procedure was repeated three times running, and the three resulting supernatant fluids were pooled (550 mL) and passed through 0.45 μm filters (Millipore Co., Bedford, MA). This fraction was designated cell wall proteinase (CWP) extract.

Ammonium Sulfate Precipitation. Solid ammonium sulfate was added to the CWP extract to a final concentration of 14%

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S0021-8561(97)00125-8 CCC: $14.00

(w/v). Insoluble material was removed by centrifugation (20000*g*, 20 min, 4 °C), and additional ammonium sulfate was added to the CWP to a final concentration of 35% w/v to precipitate the proteinase-containing material. After subsequent centrifugation, the pellet was dissolved in 24 mL of 20 mM Bis-Tris buffer, pH 6.0. This and other subsequent steps of purification were carried out at 4 °C.

Hydrophobic Interaction Chromatography (HIC). Solid ammonium sulfate (1.3 M final concentration) was added to the redissolved precipitate obtained as described above. This solution (3 mL) was injected onto a phenyl-Superose HR 5/5 (Pharmacia Biotech, Uppsala, Sweden) column, equilibrated with 1.3 M ammonium sulfate in 20 mM Bis-Tris buffer, pH 6.0. Bound proteins were eluted at 0.25 mL min^{-1} in a linear decreasing gradient of ammonium sulfate, 1.3−0.6 M in the Bis-Tris buffer. Fractions containing proteinase activity were pooled and desalted by passing them through a Sephadex G-25 PD-10 column (Pharmacia Biotech) previously equilibrated with the Bis-Tris buffer. The sample obtained was designated the HIC fraction.

Anion Exchange Chromatography. The desalted fraction (7 mL) was then applied to a Mono Q HR 5/5 (Pharmacia Biotech) column, equilibrated with the Bis-Tris buffer. Proteins were eluted at 0.5 mL min^{-1} with a linear gradient of NaCl, 0−1 M. Fractions (1 mL) were collected and immediately tested for proteinase activity. To avoid further autoproteolysis, the samples were inactivated by heating (5 min, 90 °C) and then desalted as described above, before lyophilization and electrophoretic analysis.

Measurement of Proteinase Activity. The enzyme activity was continuously measured in 50 mM sodium phosphate, pH 7.0, at 30 °C by following the hydrolysis of the substrate (1 mM) MeOsuc-Arg-Pro-Tyr-pNA (MS-Arg) (Kabi Diagnostica, Sweden) as described by Martín-Hernández *et al.* (1994). When necessary, noncontinuous measurements of proteinase activity were performed by incubation under the same conditions, after which the reaction was stopped by adding 250 μL of glacial acetic acid (80% v/v) followed by centrifugation. The activity was measured at 410 nm in a Shimadzu UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) with a CPS-240 thermostatic controller.

One unit of proteinase activity was defined as the amount of enzyme required to release 1 nmol of *p*-nitroaniline/min under the assay conditions. The molar extinction coefficient of *p*-nitroaniline was determined to be $E_{410} = 8800\ M^{-1}\ cm^{-1}$.

Protein Quantification. Protein was estimated according to the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Characterization of the Proteinase. *Effect of pH and Temperature.* The effect of pH on the proteinase activity at 30 °C was determined with an incubation mixture containing 50 μL of the HIC fraction, 400 μL of buffer with the desired pH values (0.1 M acetate, pH 4.0 and 5.0; 0.1 M sodium phosphate, pH 6.0 and 7.0; 0.1 M Tris-HCl, pH 7.5 and 8.0; 0.1 M glycine−NaOH, pH 9.0), and 50 μL of 10 mM MS-Arg in water. To estimate pH stability, the partially prepurified protein was incubated for 24 h at 4 °C in 0.1 M sodium phosphate at pH 6.0, 7.0 and 8.0.

Proteinase activity was assayed at different temperatures (20−60 °C) in buffered enzyme solutions (50 μL of HIC fraction in 400 μL of 50 mM sodium phosphate buffer, pH 7.0, and 50 μL of 10 mM MS-Arg in water). To estimate thermostability, a preincubation of enzyme solution in 50 mM sodium phosphate, pH 7.0, was carried out at different temperatures (from 25 to 60 °C) for 30 min. After cooling, residual activities were determined at 30 °C by addition of the substrate.

Influence of Chemical Reagents. The incubation mixture (450 μL) during preincubation (30 min, room temperature) contained the enzyme solution (HIC fraction), 50 mM sodium phosphate, pH 7.0, and different concentrations of specific inhibitors (protease inhibitor set, Boehringer, Mannheim, Germany) and other chemical reagents indicated in Table 2. For the determination of the residual proteinase activity, 50 μL of MS-Arg solution (10 mM) in water was added.

Specificity of the Proteinase. The relative activity of the proteinase in 50 mM sodium phosphate, pH 7.0, toward the chromophoric peptides MS-Arg (Kabi) and Suc-Ala-Glu-Pro-Phe-*p*NA (S-Glu) (Bachem, Switzerland) in the presence and absence of 1.5 M NaCl was measured as described by Martín-Hernández *et al.* (1994).

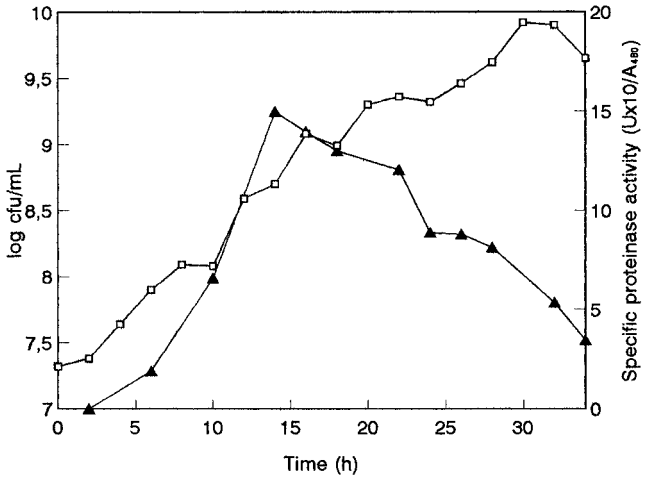

Figure 1. Growth and proteinase activity during incubation of *Lb. casei* subsp. *casei* IFPL 731 in enriched skimmed milk referred to absorbance at 480 nm after clarification by adding 0.2% EDTA, pH 12 (units × $10/A_{480}$): (□) log cfu mL^{-1}; (▲) specific proteinase activity.

Casein (α_{s1}- + β-casein) (Sigma Chemical Co., St. Louis, MO) degradation at high and low ionic strength was followed by electrophoretic analysis as described previously (Fernández de Palencia *et al.*, 1995).

Degradation of α_{s1}- and β-casein (Sigma) (final concentration of 30 mg/mL) by the partially purified proteinase was performed in 50 mM sodium phosphate, pH 7.0, at 30 °C. The reaction was stopped by adding trifluoroacetic acid (TFA) to 1.1% (v/v) final concentration to lower the pH. Hydrolysis was followed (at 0, 1, and 3 h of incubation) by reversed-phase HPLC (Zevaco and Gripon, 1988).

Degradation of fragment 1−23 of α_{s1}-casein [α_{s1}-CN(f1−23)], purified according to the procedure of Exterkate and Alting (1993), was performed in 50 mM imidazole, pH 6.5, and enzymatic hydrolysis was followed (at 0, 1, and 2 h of incubation) by reversed-phase HPLC (Exterkate *et al.*, 1991). Peak identification was based on previous results (Martín-Hernández *et al.*, 1994).

SDS−PAGE. The molecular mass of the purified proteinase was estimated by SDS−PAGE using the Mini-Protean II (Bio-Rad, Chemical Division, Richmond, VA). Electrophoresis was carried out with polyacrylamide gels of 4−25% (Ready gradient gels, Bio-Rad). The proteins were visualized by Coomassie brilliant blue R-250 (Merck, Darmstadt, Germany). For determination of M_r, high molecular mass standard proteins (MW-SDS-200 Kit, Sigma), were used.

Reversed-Phase HPLC. HPLC was carried out with a Kontron Instruments (Milan, Italy) chromatographic system using a 20 μL loop and a HI-PORE RP-318 analytical column (250 × 4.6 mm) (Bio-Rad).

RESULTS AND DISCUSSION

Production of the Proteinase. Proteinase production during 34 h of growth in skim milk is shown in Figure 1. Proteinase activity peaked at the mid-log growth phase (10^9 cfu mL^{-1}) and then declined sharply. This is consistent with the results obtained for *Lb. helveticus* CP790 by Yamamoto *et al.* (1993). It has been reported that proteinase production in lactococci is highest at the end-exponential-early-stationary phase of growth (Laan *et al.*, 1993).

Enzyme Purification. Previous work (Fernández de Palencia *et al.*, 1995) has demonstrated the strongly membrane-bound character of the CEP of *Lb. casei* IFPL 731, which could not be released using calcium-free

Table 1. Purification Scheme for the CWP of *Lb. casei* Subsp. *casei* IFPL 731

purifn step	total protein (mg)	total act. (units)	spec act. (units/mg of protein)	purifn (-fold)	yield (%)
CWP extract	724	3149	4	1	100
ammonium sulfate precipitation	135	2092	16	4	66
phenyl-Superose	0.12	844	7033	1758	27
Mono Q	0.02	175	9210	2302	6

buffer. The use of a calcium-chelating agent such as EDTA in the incubation buffer increased the enzyme release to more than 6 times the release using buffer alone. This procedure (~20% yield) was therefore used in the present work to release the CEP from the cells.

The specific activity, degree of purification, and yield obtained during purification are summarized in Table 1. After precipitation with ammonium sulfate, most of the proteinase activity appeared to be present in the 14−35% (w/v) ammonium sulfate fraction. At this stage there was 66% recovery of the total activity in 19% of the total amount of protein. After HIC, all of the activity was eluted at 0.57 M $(NH_4)_2SO_4$. In the final anion exchange chromatography step, most activity eluted at 0.22 M NaCl. This active fraction appeared as a single protein band of about 150 kDa in SDS−PAGE (Figure 2). The enzyme was purified 2300-fold with 6% recovery. However, this fraction was very unstable, losing all activity in few minutes at 4 °C. Diafiltration (30 kDa filter) of the sample showed no retention of the activity in the retentate and only very little in the permeate, suggesting the presence of active low molecular weight autoproteolytic products of the enzyme.

Contrary to the findings for lactococci by Coolbear *et al.* (1992) and Exterkate (1995), the addition of low concentrations of Ca^{2+} to the proteinase during purification did not reinforce stability. Moreover, autoproteolytic events were not prevented by the addition of other stabilizing agents such as glycerol, dithiotreitol (DTT), or ammonium sulfate.

Molecular Mass. The molecular mass of the purified proteinase was estimated to be around 150 kDa by SDS−PAGE (Figure 2). In the literature, different molecular weights have been reported for purified proteinases from lactic acid bacteria depending on the autoproteolytic fragment purified. The purified CEP of *Lactobacillus paracasei* subsp. *paracasei* NCDO 151 therefore showed two proteolytically active components, with molecular masses of 135 and 110 kDa, respectively, as determined by SDS−PAGE (Naes and Nissen-Meyer, 1992). Nevertheless, a cell wall associated proteinase of *Lb. helveticus* of low molecular mass (45 kDa) has been described by Yamamoto *et al.* (1993). On the other hand, the molecular masses reported for the CEP of *L. delbrueckii* subsp. *bulgaricus* (Laloi *et al.*, 1991) and *L. helveticus* L89 (Martín-Hernández *et al.*, 1994) were higher (170 and 180 kDa, respectively).

Further Characterization of the Proteinase. Owing to the high instability of the enzyme, the characterization studies were carried out with the partially purified HIC fraction.

Effect of pH and Temperature. The optimum pH found for hydrolysis of the substrate MS-Arg (determined at 30 °C) by the CEP of *Lb. casei* IFPL 731 was 6.0. The effect of pH 6.0, 7.0, and 8.0 on enzyme stability after incubation for 24 h at 4 °Cwas not very substantial. In all cases the residual activity was between 12 and 19%.

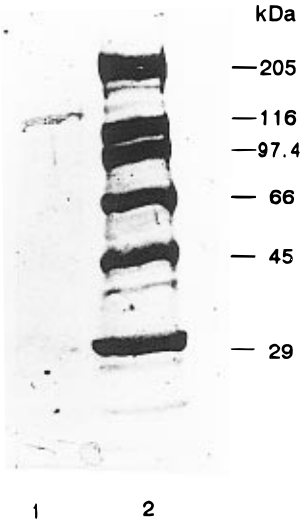

Figure 2. SDS−PAGE (4−25% gradient gel) of the purified proteinase of *Lb. casei* subsp. *casei* IFPL 731: lane 1, purified enzyme; lane 2, high molecular mass standard proteins.

Table 2. Effect of Chemical Reagents on the Proteinase Activity of *Lb. casei* Subsp. *casei* IFPL 731

reagent	concn	rel act.[a] (%)
Ca^{2+}	1 mM	141
	5 mM	136
	10 mM	137
EDTA	1 mM	103
	5 mM	88
	10 mM	102
PMSF	1mM	5
PHMB	1 mM	86
DTT	1mM	94
	5 mM	74
	10 mM	85
L-cysteine	1 mM	63
	5 mM	5
1,10-phenanthroline	1 mM	76
protease inhibitors set		
antipapain	50 μg/mL	72
bestatin	40 μg/mL	96
chymostatin	100 μg/mL	28
E64	0.5 mg/mL	113
leupeptin	0.5 μg/mL	65
pestatin	0.7 μg/mL	100
phosphoramidon	4 μg/mL	83
pefabloc	100 μg/mL	69
aprotinin	2 μg/mL	63

[a] Relative activity is referred to an untreated sample.

The optimum temperature of the enzyme was around 40 °C at pH 7.0. No major loss of enzymatic activity had been observed during preincubation at 25 °C for 30 min. However, the enzyme exhibited 50% loss of activity at 35 °C and total loss at 50 °C. High instability has also been reported by Naes *et al.* (1991) for the partially purified CEP of *Lb. casei* NCDO 151.

Effect of Specific Inhibitors. Table 2 summarizes the effect of various reagents on proteinase activity. Like the CEP of lactococci and lactobacilli, the proteinase of *Lb. casei* IFPL 731 was strongly inhibited by the serine proteinase inhibitor phenylmethanesulfonyl fluoride (PMSF) and also by chymostatin. The proteinase was not clearly affected by reducing agents such as DTT or the thiol proteinase inhibitors *p*-hydroxymercuribenzoic acid (PHMB) and E-64. In addition, cysteine showed an inhibiting effect (95%), indicating that the thiol group is not important for enzyme activity. The purified proteinase was not significantly influenced by EDTA, while calcium ions increased its activity at the experi-

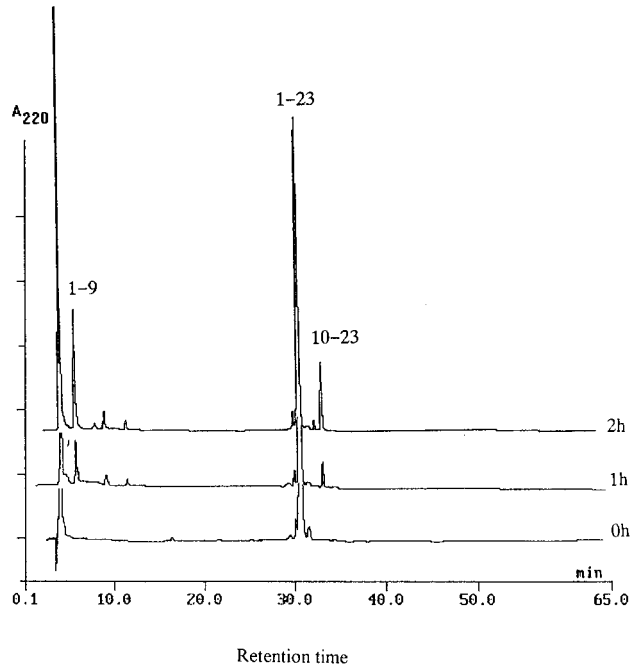

Figure 3. Reversed-phase HPLC patterns of the hydrolysis products after 1 and 2 h of incubation (30 °C) of α_{s1}-CN(f1−23) with the proteinase of *Lb. casei* subsp. *casei* IFPL 731.

mental concentrations. This could be explained by partial inhibition of the proteinase activity due to the presence of EDTA in the extraction buffer. Further addition of calcium ions could restore initial activity. In general, the agent EDTA partially inhibits the activity of lactobacilli proteinases when these are released without using it (Naes *et al.*, 1991; Yamamoto *et al.*, 1993; Martín-Hernández *et al.*, 1994).

Substrate Specificity. In line with previous results obtained using proteinase crude extracts of *Lb. casei* IFPL 731 (Fernández de Palencia *et al.*, 1995), the partially purified proteinase can be classified as an Lc-CEP $_{I/III}$ mixed-type described by Exterkate *et al.* (1993). It was highly specific toward MS-Arg substrate at low and high ionic strengths (55 and 100%, respectively), and the substrate S-Glu was only slightly hydrolyzed at high ionic strength (27%). These results were expressed as a percentage of the activity values for MS-Arg in 1.5 M NaCl. As with crude proteinase extracts, the primary proteolysis of α_{s1}- and β-caseins by the proteinase showed that β-casein was hydrolyzed more quickly than α_{s1}-casein and was completely degraded after 1 h of incubation. At high ionic strength (4% NaCl), lower degradation of both caseins was observed. The cited controversial results on the specificity of the CEPs of *Lb. casei* toward caseins (Khalid and Marth, 1990; Kojic *et al.*, 1991, 1995) suggest the existence of different proteinase variants, not only in this species but also in others such as *Lb. helveticus* (Martín-Hernández *et al.*, 1994).

Figure 3 shows HPLC patterns of the degradation products of the fragment α_{s1}-CN(f1−23) produced by the action of the proteinase during 0, 1, and 2 h of incubation at 30 °C. The specific primary action of the proteinase on peptide bond 9−10 resulted in two major fragments, f(1−9) and the complementary f(10−23). No further conversion of these fragments was observed. Specificity for the bond 9−10 has also been observed in mixed-type proteinases of lactococci (Exterkate *et al.*, 1993) and the CEP of *Lb. helveticus* (Martín-Hernández *et al.*, 1994).

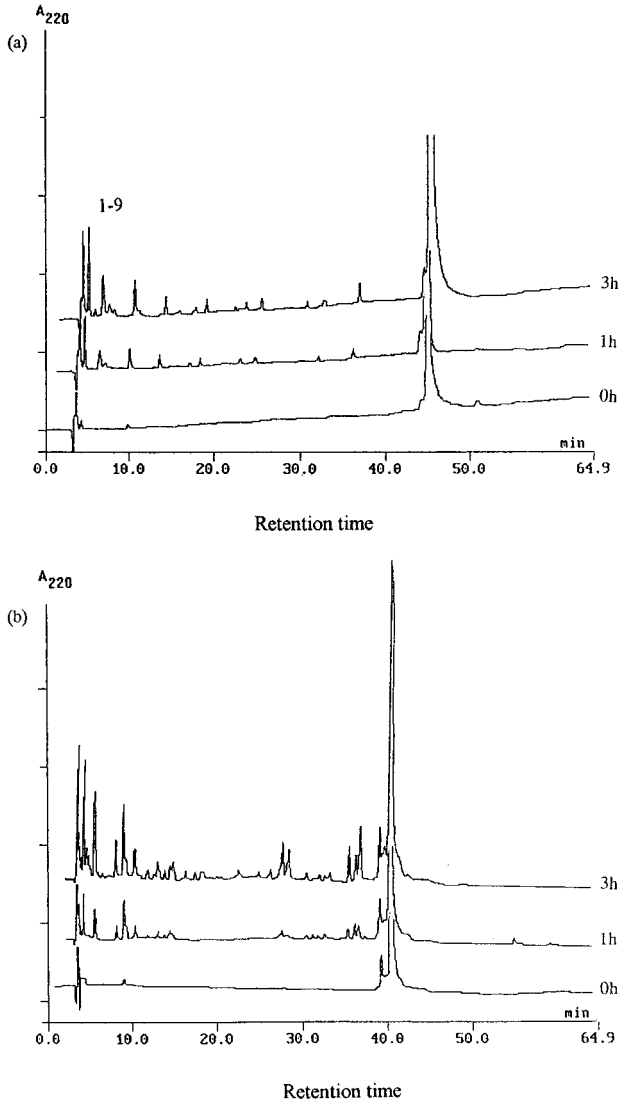

Figure 4. Reversed-phase HPLC patterns of the peptides contained in the 1.1% TFA soluble fraction obtained after 1 and 3 h of incubation (30 °C) of α_{s1}-casein (a) and β-casein (b) with the proteinase of *Lb. casei* subsp. *casei* IFPL 731.

The action of the proteinase on both α_{s1}- and β-caseins resulted in a complex pattern of TFA-soluble peptides (Figure 4). Hydrolysis was higher against β-casein than against α_{s1}-casein, and most of the hydrolysis products of the β-casein eluted at retention times between 5 and 15 min (Figure 4b). There was also a second zone of eluting fragments between 28 and 38 min. The hydrolysis products profile of the CEP against β-casein has been extensively studied for lactococci (Monnet *et al.*, 1989; Visser *et al.*, 1991; Reid *et al.*, 1991; Coolbear *et al.*, 1992; Juillard *et al.*, 1995) and to a much lesser extent for lactobacilli (Zevaco and Gripon, 1988; Yamamoto *et al.*, 1993). Some of the hydrolysis products of the β-casein can play an important role in the bitterness of cheese. The action of the proteinase on α_{s1}-casein is shown in Figure 4a. Besides other peptides, the f(1−9) fragment is the main component of the profile because of the accessibility of the proteinase of *Lb. casei* IFPL 731 to peptide bond 9−10 of casein. This fragment has also been identified as the main product of hydrolysis of the α_{s1}-casein by the *Lb. helveticus* CNR and L89 proteinase (Zevaco and Gripon, 1988; Martín-Hernández *et al.*, 1994).

The present article describes some properties of the cell envelope proteinase of *Lb. casei* IFPL 731. Unlike

lactococci proteinases, this enzyme exhibits high instability at 4 °C due to autoproteolysis and it shows a strongly membrane-bound character, so that chelating agents are necessary to release it. In addition, the Ca^{2+} ion proved ineffective as a stabilizing agent. The purified proteinase can be classified as a $CEP_{I/III}$ mixed-type variant. Further studies on its characterization are currently in progress.

ABBREVIATIONS USED

CEP, cell envelope proteinase; PMSF, phenylmethanesulfonyl fluoride; Lc-CEP, cell envelope proteinase of *Lactococcus*; Lb-CEP, cell envelope proteinase of *Lactobacillus*; CWP, cell wall proteinase; HIC, hydrophobic interaction chromatography; PHMB, *p*-hydroxymercuribenzoic acid.

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Received for review February 12, 1997. Revised manuscript received June 10, 1997. Accepted June 18, 1997.[⊗] This work was carried out under the terms of reference of research projects ALI94-0735 (Interministerial Commission for Science and Technology) and AIR 2-CT93-1531.

JF970125W

[⊗] Abstract published in *Advance ACS Abstracts,* August 1, 1997.