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Extraction and Partial Characterization of Proteolytic Activities from the Cell Surface of *Lactobacillus helveticus* Zuc2

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ABSTRACT

Proteolytic activities were extracted from a dairy *Lactobacillus helveticus* strain and partially characterized. A first cell envelope proteinase (CEP) was extracted using a high ionic strength buffer, both in the presence and in the absence of Ca^{2+}. Moreover, cell treatment by 5 *M* LiCl allowed for the selective removal of the S-layer protein and CEP, suggesting an enzyme ionic linkage to the cell envelope similar to that observed for the S-layer structure. The enzyme specificity against α_{s1}-CN (f1-23) showed unusual activity on the Lys_3-His_4 bond compared with other proteinases of the same species. A second proteinase appeared to be linked to the cell membrane because it was extractable only after membrane disgregation by detergents. Its specificity against CN fractions and α_{s1}-CN (f1-23) was different from that of the first CEP; moreover, the measured activity was lower than that of CEP.
Key words: *Lactobacillus helveticus,* cell envelope, proteinase, specificity

INTRODUCTION

Lactobacillus helveticus represents the prevailing species among the thermophilic bacteria of natural whey starter cultures, and it plays an essential role in the ripening of different cheeses, such as Italian hard cheese and Swiss-type cheese. Morishita et al. (1981) demonstrated that *L. helveticus* strains are auxotrophic for most of the AA. Therefore, because of the low free AA content, its growth in milk depends on an extensive CN breakdown by proteolytic enzymes whose activity is considered the strongest among the lactic acid bacteria (Kunji et al., 1996). For this peculiar characteristic, its use has been suggested as a starter adjunct to Cheddar cheese milk because an attenuated culture enhances the flavor development (Makdor et al., 2000). Casein breakdown results from the action of extracellular proteinase(s), and the hydrolysis products (from 4 to 18 AA) are translocated into the bacterial cell via an oligopetide transport system. Various cytoplasmatic peptidases, with partially overlapping specificities, then degrade the internalized oligopeptides into free AA (Kunji et al., 1996).

Several reports are available on the cell envelope proteinase (**CEP**) of *L. helveticus,* which display a strong heterogeneity among such enzymes (Martin-Hernandez et al., 1994; Ono et al., 1997; Hébert et al., 1999).

In some cases, an appreciable release of CEP from *L. helveticus* strains can be achieved by use of a calcium-free buffer, such as that obtained for lactococcal proteinase (Ono et al., 1997). On the contrary, CEP of the *L. helveticus* L89 and CRL 1062 strains showed a strong membrane-bound character and was poorly released in a calcium-free extraction buffer (Martin-Hernandez et al., 1994; Hébert et al., 1999). *Lactobacillus helveticus* CEP show a wide heterogeneity in the relative molecular mass (M_r) of purified enzymes (ranging from 45 to 180 kDa), immunological properties (Yamamoto et al., 1998), and CN breakdown specificity (Ono et al., 1997). Moreover, the proteolytic system of this species is considered to be useful for the production of bioactive peptides; in fact, several bioactive peptides are released from CN either during milk fermentation by strains of this genus (Matar et al., 2001; Scolari et al., 2002; Seppo et al., 2003) or by their isolated proteinases (Maeno et al., 1996).

Pederson et al. (1999) demonstrated the presence of 2 different CEP in a PrtH deletion derivative of the CNRZ 32 strain, obtained by gene replacement. Moreover, a gene (*htr*A) coding for a stress-inducible Htr-like protein from *L. helveticus* CNRZ 32 has been cloned, sequenced, and characterized (Smeds et al., 1998). This protein carries the characteristic catalytic domain of trypsin-like proteases, and the authors proposed the location of HtrA of *L. helveticus* to be on the outer surface of the plasma membrane, on the basis of the AA sequence.

This paper describes the extraction of 2 proteinases from a strongly proteolytic *L. helveticus* strain and the partial characterization of the most loosely CEP-bound enzyme.

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Table 1. Buffers used in the cell envelope proteinase extraction trials[1]

Extractant	pH	Concentration (*M*)	Addition
P_i	7.0	0.05, 0.1, 0.25, 0.75, 0.50, 1.00	—
P_i	7.0	0.5	PMSF (1 m*M*)
P_i	7.0	0.5	EDTA (20 m*M*)
MES	7.0	0.5	—
MES	7.0	0.5	$CaCl_2$ (20 m*M*)
LiCl	—	5	—

[1]P_i = phosphate buffer; PMSF = phenyl-methyl sulfonyl fluoride; MES = morpholine-ethane sulfonic acid.

MATERIALS AND METHODS

Chemicals and Chromatographic Materials

Inhibitors, lysozyme, Igepal Ca-360, and alkaline peptidase, as well as the α-CN, β-CN, and κ-CN fractions, were obtained from Sigma (Sigma Chemical Co. St. Louis, MO). The SDS molecular weight standard was from Bio-Rad Laboratories (Hercules, CA). Succinyl-Ala-Ala-Phe-7-amido-4-methylcoumarin (**AMC**), benzoyl-Pro-Phe-Arg-AMC, benzoyl-Phe-Val-Arg-AMC, and benzoyl-Val-Gly-Arg-AMC were obtained from Bachem (Bubendorf, Switzerland).

Strains and Culture Conditions

The *L. helveticus* Zuc2 used in this study was previously isolated from natural whey culture for Grana Padano cheese and identified by biochemical tests, randomly amplified polymorphic DNA (**RAPD**)-PCR analysis, and the electrophoretic mobility of X-prolyl-dipeptidyl aminopeptidase (Scolari and Vescovo, 2004). It was maintained in filter-sterilized cheese whey under liquid nitrogen vapor at the collection of the UCSC Microbiology Institute (Piacenza, Italy) and, when required, was subcultured overnight in the same medium at 44°C.

Proteinase Extraction and Purification

Lactobacillus helveticus Zuc2 cells from an overnight culture (250 mL) were harvested by centrifugation at 3,000 × *g*, 4°C, for 10 min, washed twice in 50 m*M* β-glycerolphosphate buffer, pH 7.0, and 15 m*M* $CaCl_2$ and resuspended to 1:40 of their original volume in each of the extraction buffers listed in Table 1. Bacterial cells were incubated at 37°C for 30 min under mild agitation to extract CEP, and were successively removed from suspension by centrifugation at 12,000 × *g*, 4°C, for 10 min. Supernatants were assayed for cell lysis by measuring the intracellular lactate dehydrogenase (EC 1.1.1.127) and X-prolyl-dipeptidyl aminopeptidase (EC 3.4.14.5) activities according to Scolari and Vescovo (1996) before storage at –80°C.

The proteinase extracted by 0.5 *M* phosphate (P_i) buffer, pH 7.0, was loaded (750 μL) onto a gel filtration Superdex 200 HR 10/30 column (Pharmacia Biotech, Uppsala, Sweden) previously calibrated by a gel filtration high molecular weight calibration kit (Pharmacia Biotech), and samples were eluted using 50 m*M* P_i buffer, pH 7.0, and 150 m*M* NaCl at a flow rate of 0.4 mL/min. Eluted fractions (5 mL) were monitored by a UV detector (280 nm) and assayed for proteolytic activity; the active fractions were concentrated sixfold by diafiltration on 10-kDa molecular weight cut-off centricon concentrators (Millipore, Bedford, MA) against the equilibration buffer of the next chromatography. The second purification step was performed on a MonoQ ion-exchange column (Pharmacia Biotech) equilibrated with 20 m*M* P_i buffer, pH 7.5, at a flow rate of 1 mL/min. The enzyme was eluted by a 30-min linear gradient of 0 to 0.5 *M* NaCl in the equilibration buffer, and the active fractions (containing 3.5 μg/mL of the purified enzyme) were stored at –80°C until use (CEP fractions). Moreover, after cell fractionation, the proteolytic activity extracted from the membrane fractions was subjected to the same chromatographic steps as CEP purification, but using a linear gradient of 0 to 3 *M* NaCl for MonoQ chromatography. All the above steps were performed at 4°C.

Bacterial Cell Fractionation

Washed cells from an overnight culture were obtained as described in the previous section. To obtain protoplasts and the cell wall fraction, pellets were suspended to 1:25 of their original volume in the digestion buffer and processed as described by Scolari and Vescovo (1996). Protoplasts were separated from the supernatant (cell wall fraction) by centrifugation at 1,300 × *g*, 4°C, for 10 min. To remove the high concentration of protoplasts stabilizing sucrose (17%), the cell wall fraction was subjected to dialysis for 24 h at 4°C. Pellets containing protoplasts were washed with the digestion buffer and resuspended at 1:25 of the original culture volume in digestion buffer deprived of lysozyme and saccharose. After mechanical disruption of the protoplasts by mild sonication in ice-cold 50 m*M* P_i buffer, pH 7.0, unbroken particles were selectively settled by centrifugation at 1,000 × *g*, 4°C, for 10 min; centrifugation of the supernatant at 16,000 × *g*, 4°C, for 10 min produced membrane (pellet) and cytoplasmic (supernatant) fractions.

The washed membranes were suspended to the volume of the cytoplasmic fraction using the same buffer with 1% (vol/vol) Igepal Ca-360 added, and were almost completely dissolved by stirring for 2 h at 4°C. The detergent was removed from the solubilized mem-

branes by exhaustive diafiltration on 10-kDa molecular weight cut-off centriprep concentrators (Amicon Inc., Beverly MA) using 20 m*M* P_i buffer, pH 7.5. Cell fractions were assayed for proteolytic activity.

Proteinase Characterization

Cell envelope proteinase fractions were dialyzed against each assay buffer before determination of the enzyme temperature optimum (measured at pH 7.0) and pH optimum (measured at 37°C). Proteolytic activity was measured at 30, 37, 42, and 55°C as indicated in the following sections; measures at the desired pH values (5.0 to 7.0) were performed using the same substrate in 50 m*M* citrate-P_i buffer.

The effect of inhibitors was evaluated by measuring the activity of the same fractions against caseinate at the conditions described above in the presence or absence of each protease inhibitor: 1 m*M* phenyl-methyl sulfonyl fluoride (**PMSF**), 100 μ*M* 3,4-dichloroisocoumarin, 1 m*M* pepstatin, and 10 μ*M* E-64 as a thiol-protease inhibitor.

Proteinase Specificity

Equal volumes of individual CN suspensions (20 mg/mL in 50 m*M* P_i buffer, pH 7.0) and CEP fractions (2 mL), previously diafiltered in the same buffer at one-third of their original volume, were used to determine α-CN, β-CN, and κ-CN breakdown by the purified enzyme. Samples for SDS-PAGE analysis were taken after 0, 10, 60, and 120 min of mixture incubation at 37°C.

To obtain the α_{s1}-CN (f1-23) peptide, α_{s1}-CN B was purified and the pepsin was hydrolyzed as described by Kaminogawa et al. (1986). The α_{s1}-CN (f1-23) fragment was purified from the hydrolysis mixture by means of semipreparative reversed-phase (RP)-HPLC on a C_{18} Radial-Pack cartridge (Waters Associates, Milford, MA) in a trifluoroacetic acid–CH_3CN solvent system (solvent A: 0.115% trifluoroacetic acid in water; solvent B: 0.1% trifluoroacetic acid–60% CH_3CN in water). Samples were eluted using a 60-min linear gradient from 0 to 100% solvent B and recorded at 214 nm.

The κ-CN (f106-169) glycomacropeptide fragment (**GMP**) was separated from the cheese whey by semipreparative RP-HPLC under the same conditions as used for α_{s1}-CN (f1-23) purification and monitored for the presence of sialic acid by the thiobarbituric acid reaction (Sick et al. 1990). Solvent was removed from the GMP-containing fractions by evaporation, and AA analysis was performed according to Scolari et al. (1996) following acid hydrolysis for 24 h at 110°C under vacuum in 6 *M* HCl containing 0.1% (wt/vol) phenol. The peptide was identified by comparing the AA composition with that calculated from the GMP sequence.

Purified α_{s1}-CN (f1-23) and GMP peptides were incubated (final concentration of each: 3 μg/mL) with CEP and the membrane proteinase preparations at 37°C, tracking samples after 10 and 30 min.

The specificity of CEP against the α_{s1}-CN (f1-23) and GMP fragments was analyzed by RP-HPLC separation of the degradation products according to Exterkate et al. (1991) on an ACCQ·Tag RP-C18 column, 3.9 × 150 mm (Waters Associates). Peaks were collected, dried, and identified by mass spectrometry performed on a laser desorption time-of-flight system (model G2025A; Hewlett-Packard, Palo Alto, CA). Peptides from GMP hydrolysates were identified by N-terminal sequencing and determination of the AA profile.

Proteolytic Activity Measurements and SDS-PAGE Electrophoresis

Preparations containing proteolytic activity (3.5 μg/mL for pure CEP and 0.8 μg/mL for protease from membranes) were mixed with the same volume of 1.5% (wt/vol) Na caseinate in 50 m*M* P_i buffer, pH 7.0, and 0.1% (wt/vol) NaN_3, dialyzed against the same buffer, and were incubated overnight at 37°C; the activity was measured as the increase in optical density at 340 nm (OD_{340}) after *o*-phthaldialdehyde derivatization according to Scolari et al. (1996).

To detect the presence of endopeptidase activity in the purified fraction of CEP, the dialyzed preparations were separated on native PAGE as described by Scolari and Vescovo (2004); gels were stained after incubation by 0.1 m*M* succinyl-Ala-Ala-Phe-AMC, benzoyl-Pro-Phe-Arg-AMC, benzoyl-Phe-Val-Arg-AMC, and benzoyl-Val-Gly-Arg-AMC in the presence of 0.8 U/mL of alkaline peptidase (EC 3.4.11.10), under the conditions reported by the same authors. Substrates were chosen following the indications of Christensen et al. (2003).

Sodium dodecyl sulfate-PAGE electrophoresis was carried out as described by Scolari et al. (1996) using a Mini-Protean electrophoretic cell (Bio-Rad Laboratories). Hydrolyzed CN and enzymatic activity were separated on 12 and 8% running gels, respectively, and enzyme M_r was determined by a low-range SDS molecular weight standard after Coomassie Brilliant Blue R-250 staining; GMP visualization was possible using 5% (wt/vol) TCA in 10% acetic acid as the destaining solution.

LiCl Treatments

The LiCl extract was exhaustively diafiltered by 30-kDa molecular weight cut-off centricon against Milli-Q

water (Waters Millipore, Milan, Italy) until a white precipitate was formed, which was recovered by centrifugation at 12,000 × *g* for 10 min and dissolved to the original extract volume in 50 m*M* P_i, pH 7.0. Both the supernatant and solubilized precipitate were assayed for proteolytic activity, and the specificity of the latter was determined against α_{s1}-CN (f1-23) as described above.

In addition, the proteolytic activity of either intact or LiCl-extracted cells was evaluated. For this purpose, *L. helveticus* Zuc2 cells, obtained as reported above, were treated twice with 3 *M* LiCl by mild agitation at 4°C for 20 min. Both types of cells were washed and suspended in 50 m*M* β-glycerolphosphate buffer, pH 7.0, added to 7 μg/mL of chloramphenicol as a protein synthesis inhibitor and incubated with α_{s1}-CN (f1-23) at 4°C for 30 min. The hydrolysis products were separated as described above, and their identification was based on previous results; unknown peaks [from α_{s1}-CN (f1-23)] were identified by AA analysis as reported above.

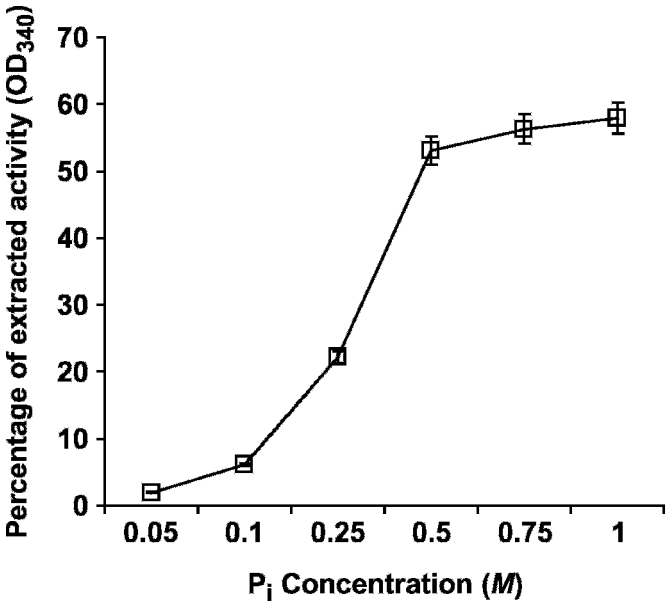

Figure 1. Percentage of the total proteolytic activity extracted from *Lactobacillus helveticus* Zuc2 cells by phosphate buffer (P_i) at increasing ionic strength. The activity is expressed as OD_{340}.

RESULTS

CEP Extraction

Lactobacillus helveticus Zuc2 was characterized by strong proteolytic activity toward CN; however, cell incubation in Ca-free buffers was ineffective in releasing such activity from the cell envelope. The recovery of CEP was enhanced by increasing the ionic strength of the extraction buffer. In particular, when using P_i extraction buffers ranging from 0.05 to 1.00 *M* (Table 1), a logarithmic relationship was evident between the release of CEP, expressed as OD_{340}, and buffer concentrations up to 0.5 *M*. Moreover, when using a buffer of still higher ionic strength, less than 40% of the enzymatic activity was retained on the treated cells (Figure 1). These results suggest the use of a 0.5 *M* P_i buffer for the CEP extraction and purification steps.

In all the extraction experiments, cell lysis was negligible because of the absence of intracellular enzyme activities (EC 1.1.1.127 and EC 1.1.1.127) in culture supernatants. To exclude a Ca^{2+}-triggered autoproteolytic event in the CEP release mechanism, *L. helveticus* Zuc2 cells were incubated with P_i buffer with 20 m*M* EDTA added, and the same extraction pattern was obtained irrespective of the presence of the additive (Figure 2, lanes 4 and 6). Comparable results were obtained using MES extraction buffer with addition of 20 m*M* $CaCl_2$. Also, an autoproteolytic release of CEP because of structural changes induced by the ionic strength of the buffer was excluded by adding 1 m*M* PMSF (Laan and Konings, 1989) to the 0.5 *M* P_i extraction buffer. In fact, the same SDS-PAGE profile was obtained in the presence and in the absence of the inhibitor (Figure 2, lanes 4 and 5). Interestingly, this profile showed a band of approximately 45 kDa, with an electrophoretic mobility corresponding to the S-layer monomer, which was also evident in the 5 *M* LiCl extract (Mozes and Lortal, 1995; Figure 2, lane 3). It is noteworthy that

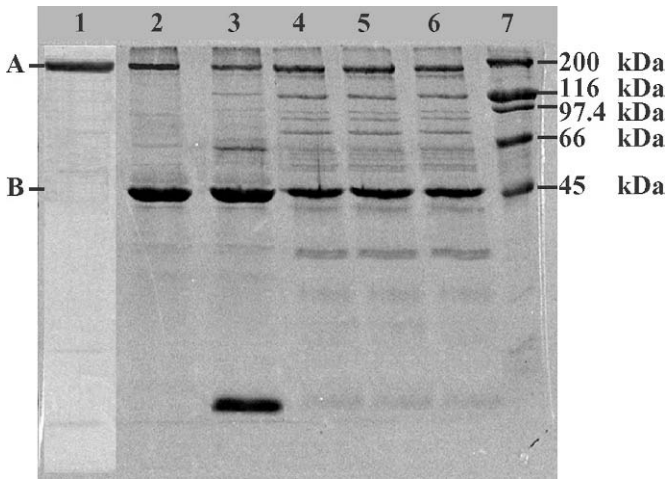

Figure 2. Sodium dodecyl sulfate-PAGE separation of various *Lactobacillus helveticus* Zuc2 cell envelope proteinase (CEP) extracts. Lane 1: purified CEP. Lanes 2 to 6: various extracts: white precipitate from dialysis (2); 5 *M* LiCl (3); 0.5 *M* phosphate buffer (P_i) (4); 0.5 *M* P_i + 1 m*M* PMSF (5); 0.5 *M* P_i + 20 m*M* EDTA (6). Lane 7: SDS molecular mass marker. A = 180-kDa proteinase; B = S-layer monomer.

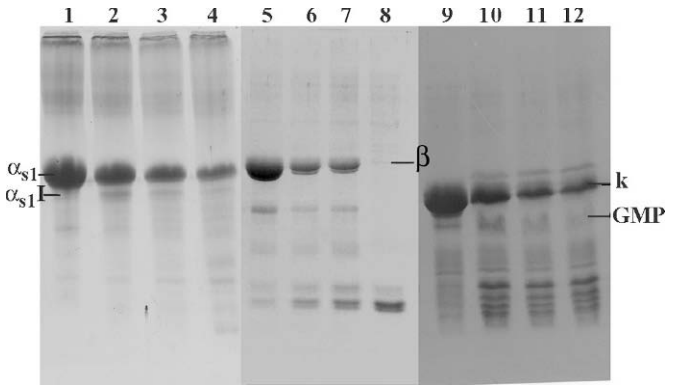

Figure 3. Digestion of α_{s1}-CN (lanes 1 to 4), β-CN (lanes 5 to 8), and κ-CN (lanes 9 to 12) by *Lactobacillus helveticus* Zuc2 cell envelope proteinase at different incubation times: 0 min, lanes 1, 5, 9; 10 min, lanes 2, 6, 10; 60 min, lanes 3, 7, 11; 120 min, lanes 4, 8, 12. α_{s1}I = α_{s1}-CN (f24-199); GMP = glycomacropeptide.

the complete removal of salt from 5 *M* LiCl extracts by dialysis produced a white precipitate, which retained the original proteolytic activity in its entirety. Sodium dodecyl sulfate-PAGE analysis of the precipitate revealed the presence of 2 main bands corresponding to the S-layer monomer (B) and to the 180-kDa proteinase (A), respectively (Figure 2, lane 2). This could suggest an ability of CEP to associate with the S-layer in *L. helveticus,* as also hypothesized by Pederson et al. (1999) for the *L. helveticus* CNRZ 32 strain.

Moreover, after complete removal of the S-layer by 3 *M* LiCl, a small amount (approximately 3 to 5%) of the total extractable proteolytic activity was still removable from the cell surface by thorough washing with 0.5 *M* P_i. The α_{s1}-CN f(1-23) fragmentation profile of this last extract was the same as that of CEP (data not shown).

Purification and Characterization of CEP

The purification scheme is shown in Table 2. The high values for specific activity and yield seem to indicate that 0.5 *M* P_i is a rather selective extractant for CEP, thus allowing for a relatively simple purification process. In fact, the same purification steps permitted only a partial purification of membrane-associated proteinase (**MAP**) from the membranes, giving a very low yield as well as very low specific activity.

The S-layer associated with CEP, extracted with 0.5 *M* P_i buffer, eluted from gel filtration chromatography between thyroglobulin (669 kDa) and ferritin (440 kDa), indicating an approximate native M_r of about 560 kDa. Fractions containing proteolytic activity were eluted by subsequent ion-exchange chromatography at an ionic strength of 0.2 *M* NaCl. On SDS-PAGE they showed a prevailing intense band of 180 kDa M_r (Figure 2, lane 1) that was associated with CEP, thus suggesting a trimeric aggregation of the extracted enzyme, possibly through the W domain of the molecule. The incubation of native gels of purified CEP fractions with the 4 endopeptidase substrates did not reveal the presence of red bands indicating the presence of such an intracellular enzyme class.

The optimal pH of CEP at 37°C is approximately 6, according to values from the literature (Ono et al., 1997). The optimal temperature at pH 7.0 was 44°C, and the enzyme maintained 40% of the activity when incubated for 2 h at 60°C. Inhibition of the enzyme was complete and irreversible with either 1 m*M* PSMF or 100 μ*M* 3,4-dichloroisocoumarin; 1 m*M* pepstatin gave an inhibition of approximately 10%, but the enzyme activity was almost completely restored after inhibitor removal. No inhibition occurred with the thiol–proteinase inhibitor E-64, thus excluding the presence of a cysteine group in the active site.

CEP Specificity

Hydrolysis of the CN Fractions. The specificity of CEP on α_{s1}-CN, β-CN, and κ-CN is reported in Figure 3. The enzyme showed intense activity toward β-CN even from the first 10 min of incubation, producing a substantial reduction in the corresponding band. Within this time, a broad band appeared in the hydrolytic profile of κ-CN that had the characteristic shape and electrophoretic mobility of the purified GMP; it was also possible to observe the formation of a fragment with electrophoretic mobility corresponding to the α_{s1}I peptide in the profile of α_{s1}-CN hydrolysates, thus indicating the specificity of the enzyme for the Arg_{22}-Phe_{23} bond of α_{s1}-CN as well as for the Phe_{105}-Met_{106} bond of κ-CN. Both fragments were subsequently partially degraded. At the end of incubation, all the fractions were thoroughly hydrolyzed, and the β-CN was completely digested.

Specificity Against α_{s1}-CN (f1-23) and GMP. A deeper knowledge of the specificity of CEP was obtained by analyzing the hydrolysis products of both α_{s1}-CN (f1-23) and GMP. Reversed-phase HPLC digestion profiles of α_{s1}-CN (f1-23) after 10 and 30 min of incubation, respectively (Figures 4A and 4B), showed a complex mixture of hydrolysis products as well as almost complete substrate degradation within the first 10 min of incubation, confirming the strong activity demonstrated by the enzyme toward the CN fractions. This digestion profile was also obtained by using the white precipitate from the LiCl extraction, but in a shorter incubation time (data not shown).

The accumulation of α_{s1}-CN (f1-9), (f10-16), and (f17-23) fragments in the first 10 min of incubation could

Table 2. Summary of the purification of cell envelope proteinase (CEP) and membrane-associated proteinase (MAP) from *Lactobacillus helveticus* Zuc2

Step	Total activity,[1] ΔOD_{340}	Total protein, mg	Specific activity, ΔOD_{340}/mg	Purification, fold	Yield, %
			(CEP)		
Crude extract	3.09	166	1.86×10^{-2}	1.00	100
Gel filtration chromatography	2.47	24	10.29×10^{-2}	5.53	79
Ion-exchange chomatography	1.03	0.105	9.81	527.42	33
			(MAP)		
Solubilized membranes	3.25	1,845	1.76×10^{-3}	1.00	100
Gel filtration chromatography	2.08	647	3.21×10^{-3}	1.82	35
Ion-exchange chomatography	0.45	37	1.22×10^{-2}	6.93	2

[1]Activity is expressed as variation in OD_{340} (optical density at 340 nm) after 12 h of incubation.

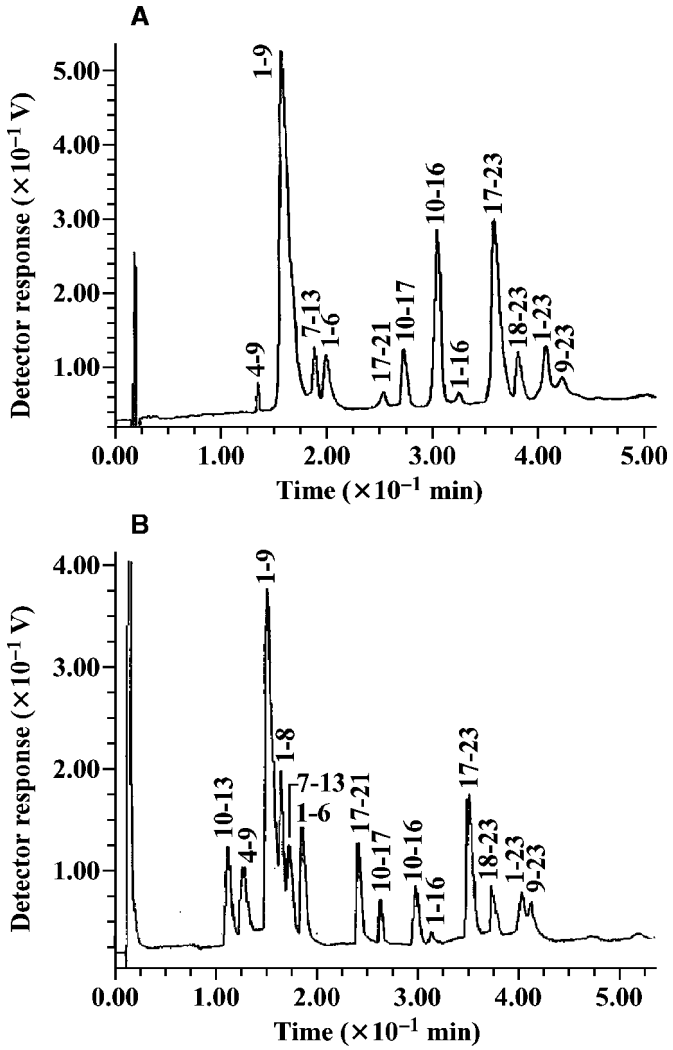

Figure 4. Reversed-phase HPLC patterns of α_{s1}-CN (f1-23) hydrolysis products after 10 min (A) and 30 min (B) of incubation at 37°C with purified *Lactobacillus helveticus* Zuc2 cell envelope proteinase.

suggest Gln_9-Gly_{10} and Leu_{16}-Asn_{17} bonds as being the primary cleavage sites. Accordingly, the peptide α_{s1}-CN (f1-16) released from splitting of the latter bond could be readily converted to the α_{s1}-CN (f1-9) and (f10-16) fragments, because the amount of α_{s1}-CN (f1-16) in the RP-HPLC profile was consistently low at both incubation times. Similarly, the fragment α_{s1}-CN (f10-23), containing the sensitive Leu_{16}-Asn_{17} bond, could be degraded to the peptides α_{s1}-CN (f10-16) and (f17-23), because it was not detectable after 10 min of incubation.

Moreover, it is possible to speculate that within the first 10 min, a small amount of the substrate was hydrolyzed at the Asn_{17}-Glu_{18} bond, releasing α_{s1}-CN (f18-23) and (f10-17), whereas the appearance of the α_{s1}-CN (f1-6) and (f7-13) peptides could suggest a similar sensitivity of the Ile_6-Lys_7 and Gln_{13}-Glu_{14} bonds to CEP activity. One of the possible degradation products of these peptide bond cleavages, the tetra peptide Glu_{14}-Val_{15}-Leu_{16}-Asn_{17}, was not detectable, probably because it did not bind to the column (Oberg et al., 2002).

Among the probable secondary hydrolytic reactions (after 30 min), the conversion of α_{s1}-CN (f1-9) at the sites Lys_3-His_4, Ile_6-Lys_7, and His_8-Gln_9 could explain the accumulation of the α_{s1}-CN (f 4-9), (f1-8), and (f1-6) fragments, whereas α_{s1}-CN (f10-17) and (f10-16) degradation (at the Gln_{13}-Glu_{14} bond) produced mainly α_{s1}-CN (f10-13).

The accumulation of α_{s1}-CN (f17-21) might originate from cleavage of the Leu_{21}-Arg_{22} bond of α_{s1}-CN (f17-23). The charged α_{s1}-CN (f22-23) and (f1-3) fragments probably eluted early in the chromatogram and were thus not detectable.

Amino acid analysis of the purified GMP showed more than 89% identity with the calculated composition, except for Thr, Leu, and Gly (70, 62, and 79%, respectively). Moreover, the contents of Tyr, Phe, His, and Arg were negligible, allowing GMP to be considered pure.

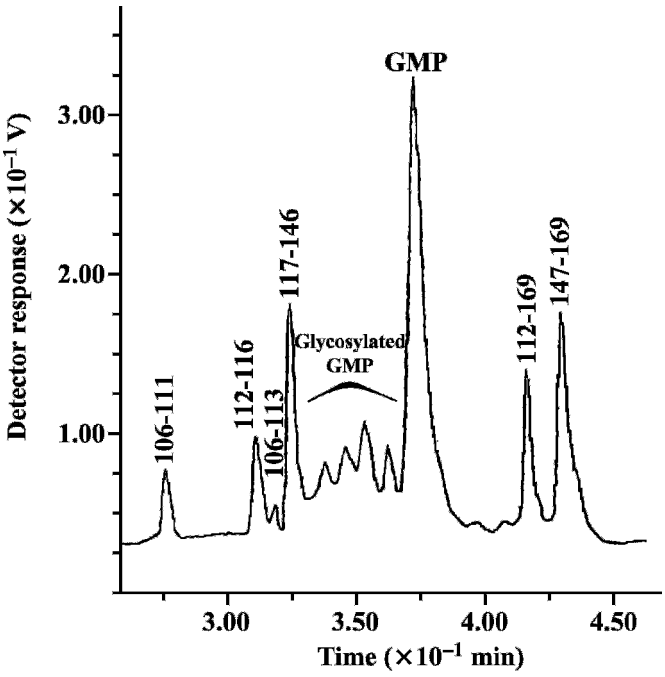

Figure 5. Reversed-phase HPLC patterns of GMP hydrolysis products after 30 min of incubation at 37°C with purified *Lactobacillus helveticus* Zuc2 cell envelope proteinase. GMP = undigested glycomacropeptide (not glycosylated fraction); glycosylated GMP = undigested glycomacropeptide (glycosylated fractions).

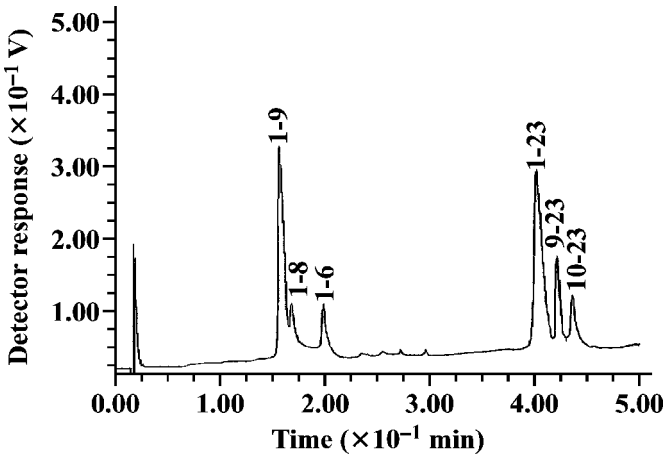

Figure 6. Reversed-phase HPLC profile of α_{s1}-CN (f1-23) hydrolysis products after 30 min of incubation with *Lactobacillus helveticus* Zuc2 cells treated by 3 *M* LiCl.

The incubation of GMP with CEP up to 30 min did not produce a spectrum of hydrolyzed peptides as wide as that obtained from α_{s1}-CN (f1-23) hydrolysis, because approximately 70% of the substrate (GMP and glycosylated GMP fractions) remained undigested (Figure 5). The main peptide fragments detected in the reaction mixture were κ-CN (f106-111), (f106-113), (f112-116), and (f117-146) as well as the carboxy-terminus (f112-149) and (f147-169) (Figure 5), thus suggesting that Lys_{111}-Lys_{112}, Lys_{116}-Thr_{117}, and Leu_{146}-Glu_{147} were the sole sensitive bonds. A similar degradation profile was obtained by Sick et al. (1990) during GMP digestion by tripsin.

Cell Fractionation and Partial Characterization of Membrane-Fraction Proteinase. Cell lysis during the protoplastization step, expressed as the percentage of intracellular lactate dehydrogenase released, was less than 5% of total cells. Cell fractionation demonstrated that proteolytic activity was partitioned between the membrane (46%) and the cytoplasmatic (17%) fractions, respectively, whereas the remaining 37% was associated with the cell wall fraction. This last percentage appeared to be in contrast to that obtained for CEP proteinase activity extracted by 0.5 *M* P_i. A possible explanation could be the environmental differences in residual activity measured on bacterial cells after P_i buffer treatment and those detected on free enzymes in the membrane fractions. Moreover, enzyme autodigestion is highly probable because of the long dialysis process of the cell wall fraction.

More than 80% of the activity in the membrane fraction was not extractable by 0.5 *M* P_i but only after membrane solubilization by the treatment with Igepal CA-360, thus suggesting an enzyme anchorage to the membrane. The enzyme contained in the solubilized and dialyzed membrane fraction was eluted from the anion-exchange MonoQ column at 3.0 *M* NaCl of the gradient; this could suggest that the MAP is distinct from the CEP observed in the 0.5 *M* P_i extraction buffer. Approximate M_r of MAP was about 215 kDa, as estimated by gel filtration chromatography. The 2 chromatographic steps mentioned permitted only partial purification of MAP from the membrane fraction, so in this preliminary study, detection of the enzyme M_r on SDS-PAGE was not possible.

Casein degradation by the partially purified MAP was much lower than that observed for the purified CEP, showing no evident specificity against the 3 CN fractions. Also, unlike CEP, the degradation of α_{s1}-CN and κ-CN by MAP did not produce the respective α_{s1}-I and GMP fragments (data not shown).

LiCl-Treated Cells. The RP-HPLC profile of α_{s1}-CN (f1-23) after 30 min of incubation with LiCl-treated whole cells is presented in Figure 6. The hydrolytic pattern is completely different from that produced by untreated cells, which substantially reproduces the specificity profile of CEP (data not shown). Only the His_8-Gln_9 and Gln_9-Gly_{10} bonds were cleaved at the assay conditions, resulting in a lower proteolytic activity of treated cells compared with untreated ones. The

fragment α_{s1}-CN (f1-8) was further hydrolyzed at the Ile_6-Lys_7 bond.

DISCUSSION

Lactobacillus helveticus Zuc2 seems to carry 2 different proteinases, showing 2 distinct cell envelope anchors. The presence of 2 cell surface proteinases in *L. helveticus* strains was first suggested by Pederson et al. (1999).

CEP

The inability of Ca-free buffers to remove CEP was also reported by Martin-Hernandez et al. (1994) for the *L. helveticus* L89 strain. A substantial release of CEP from *L. helveticus* Zuc2 was achieved only by extracting buffer at an ionic strength higher than 250 m*M*; such a value is reported as the limit beyond which electrostatic interactions are maximally weakened (Dill, 1990). The use of a specific inhibitor during the release process allowed us to exclude an autoproteolytic event promoted by conformational changes, possibly consequent to increasing the ionic strength of the extractant. Moreover, the stabilizing effect of the Ca^{2+} ion reported for the lactococcal CEP anchor (Laan and Konings, 1989) was excluded for the *L. helveticus* Zuc2 CEP, which is extractable in the presence of high $CaCl_2$ concentrations. The same results were also observed for CEP release from *L. acidophilus* BGRA 43 and *L. delbrueckii* ssp. *bulgaricus* $BGPF_1$ (Fira et al., 2001). These considerations and the low recovery of the proteinase in the cell wall fraction suggest the absence of the LPXTG sorting motif, which leads to the covalent linkage of lactococcal cell-wall proteinase (PrtP) to the peptidoglycan (Navarre and Schneewind, 1999), whose lack was observed by Pederson et al. (1999) and Germond et al.(2003) in proteinase PrtH from *L. helveticus* CNRZ 32 and PrtB proteinase from *L. delbrueckii* ssp. *bulgaricus* NCDO 1489, respectively, causing the authors to hypothesize a cell wall attachment of the CEP by electrostatic forces.

The reassociation of proteinase with S-layer monomers during the exhaustive dialysis of the 5 *M* LiCl extract supports the hypothesis of an S-layer involvement in the anchorage of the enzyme to the cell surface. S-Layer proteins could be the adhesion site for the CEP, as previously reported for a high M_r amylase in *Bacillus stearothermophilus* DSM 2358 (Egelseer et al., 1995), subtilase from *Bacillus* and *Clostridium* spp. (Schmidt et al., 1995), and pullulanase in *Thermoanaerobacterium thermosulfurigenes* EM1 (Matuschek et al., 1994). However, this is in contrast with the observation that a small amount (5%) of CEP is released by thoroughly washing *L. helveticus* Zuc2 cells in 0.5 *M* P_i buffer also after the complete removal of the S-layer by 3 *M* LiCl. Alternatively, both CEP and S-layer proteins could be linked to the underlying peptidoglycan or to the secondary cell-wall polymers by electrostatic interactions (Sara and Sleytr, 2000) through homologous sequences. In fact, Pederson et al. (1999) demonstrated that the W domain of proteinase PrtH from *L. helveticus* CNRZ 32 is homologous (up to 33%) to the C-terminal domain of the S-layer protein family of *Lactobacillus,* thus suggesting its possible role in cell-wall binding by a mechanism similar to S-layer protein anchorage (Boot et al., 1995).

The pH and the temperature optima were in accordance with those reported for *L. helveticus* CEP (Martin-Hernandez et al., 1994; Ono et al., 1997). The retained activity at 60°C assumes a practical usefulness, because the *L. helveticus* Zuc2 strain belongs to the starter microbiota of Grana cheese, whose technology requires a cooking temperature of approximately 55°C.

The complete inhibition obtained by PMSF includes CEP in the serine proteinase class. Moreover, reversible inhibition of pepstatin was less than 10%, suggesting that the enzyme does not belong to the aspartyl protease family, although some enzymes of this type are not influenced by this inhibitor—this in spite of its ability to produce GMP and α_{s1}-I peptides, which are more typically produced by the action of aspartyl proteases on Phe_{105}-Met_{106} and Arg_{22}-Phe_{23} bonds of κ- and α_{s1}-CN, respectively. However, a high susceptibility of the former bond to the lactococcal serin-type proteinase was also reported by Kunji et al. (1996). The relative accumulation of such a peptide in the first step of κ-CN hydrolysis (Figure 3) appears to be consistent with the resistance of the isolated GMP to the CEP activity (Figure 5).

Cell envelope proteinase specificity resembles that of proteinase PrtP I, primarily degrading β-CN, and to a lesser extent κ-CN and α_{s1}-CN, whereas the MAP specificity cannot be assigned to either the P I or the P III type (Kunji et al. 1996). The behavior of both the proteinases against α_{s1}-CN was different from that of *L. helveticus* CRL 1062, which preferentially digests this protein (Hébert et al., 1999).

Cell envelope proteinase cleaves α_{s1}-CN (f1-23) differently from other *L. helveticus* proteinases (Kunji et al., 1996; Ono et al., 1997; Pederson et al., 1999; Oberg et al., 2002). In fact, *L. helveticus* Zuc2 CEP hydrolyzes all the cleavage sites of all the proteinases of the same species, and it is also active on the Lys_3-His_4 bond in the peptide amino-terminus domain. Such a bond was not even split by the CEP of the strain *L. helveticus* L 89, isolated from Grana cheese whey (Martin-Hernandez et al., 1994), as was the strain used in this study.

The amino-terminus region of α_{s1}-CN [α_{s1}-CN (f1-9)] is hydrolyzed only by intracellular postproline $PepO_2$ and $PepO_3$ endopeptidases from *L. helveticus* CNRZ 32, at the Pro_5-Ile_6 bond, under cheese conditions (Sridhar et al., 2005); moreover, those authors demonstrated that the Lys_3-His_4 and Lys_7-His_8 bonds of the same peptide were sensitive to the action of endopeptidase PepE. This underlines the unique specificity of *L. helveticus* Zuc2 CEP.

The low affinity of CEP toward α_{s1}-CN seems to be in contrast with the high activity observed on the α_{s1}-CN (f1-23) substrate, probably resulting from a different accessibility between the free Arg_1-Phe_{23} sequence and the whole protein. In fact, an altered cleavage in the Arg_1-Phe_{23} sequence of α_{s1}-CN compared with the free α_{s1}-CN (f1-23) peptide was observed previously (Martin-Hernandez et al., 1994).

MAP

The resuspension of the cell membrane fraction in Ca^{2+} free buffer did not produce an appreciable release of MAP, thus demonstrating its resistance to autoproteolysis in situ. The use of a membrane destructuring detergent to release MAP suggests the strong association of the enzyme with the membrane structure, as previously hypothesized for *L. helveticus* L89 (Martin-Hernandez et al., 1994) and *L. helveticus* CRL 1062 (Hébert et al., 1999).

The hydrolyzing activity toward whole CN fractions as well the lack of sensitive bonds in the amino-terminus of the α_{s1}-CN (f1-23) peptide clearly distinguished MAP from the endopeptidases previously identified in *L. helveticus* (Fenster et al., 1997; Sridhar et al., 2005).

The stronger activity of CEP compared with that of MAP observed in intact cells could be the result of either the observed lower enzyme activity or a more peripheral location of the former, but also a difference in the specific activity.

Work is in progress to confirm the presence of the second proteinase in the *L. helveticus* Zuc2 strain and to better characterize it in order to establish the role of the 2 different proteinases during the growth of such species in cheese whey. Also, the simple extraction method used in this research will be applied to selectively recover the CEP from different *L. helveticus* strains, with the aim of checking the correlation between the ability of proteinases to produce angiotensin-converting enzyme–inhibiting peptides (or antihypertensive peptides, or both) from CN and determine their substrate specificity.

CONCLUSIONS

The results of this study suggest the presence of 2 different proteinases on the cell surface of the *L. helveticus* Zuc2 strain, although further evidence should be acquired to better characterize the MAP. The different mechanisms of enzyme linkage to the cell surface we observed allow for selective extraction of the 2 proteinases.

In particular, because of the ability, demonstrated elsewhere, for *L. helveticus* whole cells to produce bioactive (antihypertensive) peptides after CN digestion, the possibility of using an easy, discriminative extraction method for the cell envelope–anchored enzyme (CEP) constitutes an important prerequisite for obtaining a deeper knowledge of the role of the enzyme involved in production of the bioactive (antihypertensive) peptides.

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