Appl Microbiol Biotechnol
DOI 10.1007/s00253-014-6258-6

Characterization of the mature cell surface proteinase of *Lactobacillus delbrueckii* subsp. *lactis* CRL 581

Josefina M. Villegas · Lucía Brown · Graciela Savoy de Giori · Elvira M. Hebert

Received: 21 October 2014 / Revised: 18 November 2014 / Accepted: 20 November 2014

Abstract The cell envelope-associated proteinase (CEP) of *Lactobacillus delbrueckii* subsp. *lactis* CRL 581 (PrtL) has an essential role in bacterial growth, contributes to the flavor and texture development of fermented products, and can release bioactive health-beneficial peptides during milk fermentation. The genome of *L. delbrueckii* subsp. *lactis* CRL 581 possesses only one gene that encodes PrtL, which consists of 1924 amino acids and is a multidomain protein anchored to the cell via its W domain. PrtL was extracted from the cell under high ionic strength conditions using NaCl, suggesting an electrostatic interaction between the proteinase and the cell envelope. The released PrtL was purified and biochemically characterized; its activity was maximal at temperatures between 37 and 40 °C and at pH between 7 and 8. Under optimal conditions, PrtL exhibited higher affinity for succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide than for succinyl-alanyl-glutamyl-prolyl-phenylalanine-*p*-nitroanilide, while methoxy-succinyl-arginyl-prolyl-tyrosyl-*p*-nitroanilide was not degraded. A similar α- and β-casein degradation pattern was observed with the purified and the cell envelope-bound proteinase. Finally, on the basis of its specificity towards caseins and the unique combination of amino acids at residues thought to be involved in substrate specificity, PrtL can be classified as a representative of a new group of CEP.

Keywords Lactic acid bacteria · Proteinase · Casein · Proteolytic activity · Caseinolytic specificity

J. M. Villegas · L. Brown · G. Savoy de Giori · E. M. Hebert (✉)
Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, 4000 San Miguel de Tucumán, Argentina
e-mail: ehebert@cerela.org.ar

Introduction

Lactobacillus (L.) delbrueckii subsp. *lactis* is a homofermentative thermophilic lactic acid bacterium (LAB) widely used as starter cultures in several dairy fermentation processes, such as fermented sour milks and Swiss- and Italian-type cheeses. Comparative genome analysis of lactobacilli revealed that dairy LAB such as *L. delbrueckii* subsp. *lactis*, *L. delbrueckii* subsp. *bulgaricus*, and *Lactobacillus helveticus* have lost the majority of their amino acid biosynthetic genes (Callanan et al. 2008; Liu et al. 2010; van de Guchte et al. 2006) and therefore, depend on exogenous nitrogen sources for optimal growth (Hebert et al. 2004, 2008). The concentration of essential amino acids in milk is very limited. Thus, the proteolytic system of LAB is crucial to supply bacteria with essential amino acids from caseins during growth in milk, thus ensuring successful fermentation. This proteolytic system consists in a cell envelope-associated proteinase (CEP), specialized transport systems to allow uptake of the resulting peptides, and several intracellular peptidases which degrade peptides to amino acids (Hebert et al. 2008; Savijoki et al. 2006). The CEP plays a key role in this process since it is responsible for the first step of casein breakdown (Liu et al. 2010; Savijoki et al. 2006). This protease also contributes to the flavor and texture development of fermented products (Savijoki et al. 2006) and can release bioactive health-beneficial peptides during milk fermentation (Hayes et al. 2007; Hebert et al. 2010; Saavedra et al. 2013).

The CEP of lactococci and, to a lesser extent, those of lactobacilli have been subject to extensive biochemical and genetic characterization (Liu et al. 2010; Pescuma et al. 2013; Savijoki et al. 2006). On the basis of degradation patterns of α_{s1}-, β-, and κ-caseins, two proteinase specificity classes have initially been described in lactococci, CEP_I, and CEP_{III} (Exterkate et al. 1993). The primary substrate of CEP_I-type enzymes is β-casein and, to a lesser extent, κ-casein, while

Published online: 09 December 2014

CEP_{III}-type enzymes hydrolyze α_{s1}-, β-, and κ-casein (Kunji et al. 1996). In general, CEP are synthesized as preproteins of ~2000 residues, and comparative sequence analyses allowed the prediction of several functional domains (Siezen 1999). From the N-terminus, these domains include a pre-pro domain (PPro), corresponding to a typical signal sequence, followed by a prosequence that is removed by autocatalytic processing, a catalytic protease domain (PR) with a small inserted (I) domain, a domain A of unknown function, a domain B with probably a stabilizing role, a helical spacer (H) domain, a hydrophilic cell wall spacer or attachment domain (W), and a cell wall anchor domain (AN), characterized by a sorting signal (LPxTG) that covalently anchors the proteinase to the cell envelope (Navarre and Schneewind 1994; Siezen 1999). Not all domains are present in every CEP. For instance, differences in the C-terminal region among several CEP raise the possibility of another anchoring mechanism to the cell envelope. CEP of *Lactococcus* (*Lc.*) *lactis* (PrtP), *Lactobacillus paracasei* (PrtP), *Lactobacillus rhamnosus* (PrtR), and *Streptococcus* (*S.*) *thermophilus* (PrtS) possess the AN domain, suggesting covalent attachment of the proteinase to the cell wall (Savijoki et al. 2006). On the other hand, this AN domain is absent in CEP of *L. delbrueckii* subsp. *bulgaricus* (PrtB) and *L. helveticus* (PrtH), and these seem to be attached to the cell envelope through their W domain (Savijoki et al. 2006; Siezen 1999). The W domain of PrtB is rich in lysine residues (~32 %), suggesting that binding occurs through electrostatic interactions (Gilbert et al. 1996; Siezen 1999).

In LAB, calcium-free buffer incubation was the most used method to release CEP from the cell (Exterkate 1995; Martín-Hernández et al. 1994; Pederson et al. 1999; Tsakalidou et al. 1999). However, some thermophilic lactobacilli proteinases are not released by this treatment (Espeche Turbay et al. 2009; Laloi et al. 1991; Martín-Hernández et al. 1994) and can be extracted using detergent and chaotropic agents or enzymes such as lysozyme. For instance, CEP of *L. helveticus*, *Lactobacillus acidophilus*, or *L. delbrueckii* subsp. *bulgaricus* were removed from the cell envelope by using NaOH, urea, lysozyme, detergent (Nonidet P-40 or SDS), sonication, or thermal shock (Jarocki et al. 2010; Laloi et al. 1991; Martín-Hernández et al. 1994; Stefanitsi et al. 1995), while the CEP from *L. helveticus* Zuc2 was extracted using 5 M LiCl (Scolari et al. 2006).

In the last few years, significant advances on *L. delbrueckii* subsp. *lactis* CRL 581 proteolytic system were carried out. This strain, isolated from a homemade Argentinian hard cheese, possesses a CEP, named PrtL, which is able to release a series of bioactive health-beneficial peptides (i.e., anti-inflammatory, antihypertensive, and phosphopeptides) from α- and β-caseins (Espeche Turbay et al. 2012; Hebert et al. 2008; Villegas et al. 2014). Considering the industrial importance of CEP and the health-promoting attributes of CRL 581 strain in the development of novel functional foods, the aim of this work was to carry out the biochemical characterization of PrtL. Sequence analysis of the PrtL-encoding gene revealed that PrtL is a member of the subtilisin-like serine proteinases. For the first time, the CEP from *L. delbrueckii* subsp. *lactis* CRL 581 was released by an alternative method using 1 M NaCl, demonstrating that PrtL is non-covalently attached to the cell envelope. This releasing method allowed us to characterize the kinetics properties of the purified PrtL and to compare its caseinolytic specificity to that of the attached PrtL.

Materials and methods

Microorganisms, media, and growth conditions

L. delbrueckii subsp. *lactis* CRL 581, *L. helveticus* CRL 1062, *Lc. lactis* CRL 1195, and *S. thermophilus* CRL 1185 strains belonging to the culture collection of the Centro de Referencia para Lactobacilos (CERELA-CONICET, Argentina) were used. In this study, a previously formulated chemically defined medium (CDM) containing 5 mM $CaCl_2$ (CDM-Ca) was used (Hebert et al. 2004). CDM-Ca (pH 6.5) was prepared from concentrated individual stock solutions which were stored at 4 °C after filtration, except for the cysteine solution which was freshly prepared. All amino acids, vitamins, purines, pyrimidines, and inorganic salts used were of analytical grade (Sigma).

Working cultures of lactobacilli strains were grown twice in MRS broth (Biokar Diagnostics) at 37 °C for 16 h, while *Lc. lactis* CRL 1195 and *S. thermophilus* CRL 1185 were grown in LAPTg (1 % yeast extract, 1.5 % peptone, 1 % tryptone, 0.1 % Tween 80, 1 % glucose) at 30 and 37 °C, respectively, for 16 h. To eliminate carryover nutrients, cells were harvested by centrifugation at 8000×g for 15 min, washed twice in sterile 0.85 % (wt vol^{-1}) saline solution, and resuspended in this solution to the original volume. This cell suspension was used to inoculate the CDM-Ca at an initial optical density at 560 nm (OD_{560}) of 0.1.

Release of PrtL from the cell envelope

Cells grown in CDM-Ca medium were harvested by centrifugation (8000×*g* for 15 min at 4 °C) at the exponential growth phase (OD_{560}=0.80), washed twice with 0.85 % (wt vol^{-1}) saline supplemented with 10 mM $CaCl_2$, and resuspended to a final OD_{560} of approximately 20 in 20 mM Tris–HCl buffer (pH 7.5) containing either 5 mM $CaCl_2$, or NaCl (0.5, 1, and 3 M) (Villegas et al. 2011). Cells were also treated with 20 mM Tris–HCl buffer (pH 7.5) without $CaCl_2$ addition (calcium-free buffer). Samples were incubated during 30 min at room temperature and then centrifuged at 8000×*g* for 15 min. The supernatant was stored at 4 °C, and the pellet

was washed, resuspended in 20 mM Tris–HCl buffer at the original volume, and stored at 4 °C. Supernatant and pellet fractions were subjected to proteolytic activity and SDS-PAGE analyses. In order to biochemically characterize PrtL, the 1 M NaCl supernatant fraction of *L. delbrueckii* subsp. *lactis* CRL 581, obtained as previously indicated, was filtered through a 100-kDa membrane (100,000 MWCO, Amicon, Millipore) according to the manufacturer's instruction, yielding a partially purified PrtL.

SDS-PAGE and MALDI-TOF analyses

SDS-PAGE was performed as described by Laemmli (1970) on vertical slab gels. Resolving and stacking gels contained 8 and 4 % acrylamide, respectively. Electrophoresis was run at 10 mA for 5 h, and the proteins were visualized by staining with Coomassie Brilliant Blue G-250 (Candiano et al. 2004). Molecular masses were estimated using the linear relationship between the log of the molecular weight of the standards (Promega) and relative mobility (r D 0:99). Individual bands on the SDS-PAGE were excised from the gels and subjected to mass spectrometry analyses carried out by CEQUIBIEM (Facultad de Ciencias Exactas y Naturales, UBA, Argentina). Protein identity from peptide mass fingerprints was determined by the MASCOT program (Matrix Science Inc. http://www.matrix sciende.com/search-form-select.html). Fragmentation was carried out with more intense MS peaks (MS/MS).

Proteinase activity assay

Proteolytic activity was measured at 37 °C with the chromogenic substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide (S-Ala; Sigma), as described by Espeche Turbay et al. (2009). One unit of proteinase was defined as the amount required to liberate 1 nmol of nitroaniline per minute; specific activity was expressed as proteinase units per milligram of protein. Protein concentration was determined using a protein assay according to the instructions of the manufacturer (Bio-Rad). Cell lysis was determined by following the release of either lactate dehydrogenase (Thomas 1975) or lysyl-aminopeptidase using L-lysine *p*-nitroanilide as substrate as described previously (Hebert et al. 2002).

Effect of temperature and pH on proteinase activity

The effect of temperature on the activity of the purified PrtL was determined over a range of temperature from 20 to 70 °C and at pH 7.0. The enzyme was incubated in the reaction buffer with the chromogenic substrate S-Ala for 10 min at the corresponding temperature. The effect of pH on PrtL activity was determined with the S-Ala substrate at 37 °C for 10 min in a range of pH from 4 to 9 with the following 50 mM buffers: sodium acetate, pH 4.5 to 5.5, sodium phosphate, pH 6.5 to 8.0, and Tris–HCl, pH 9.

Determination of kinetics parameters

The specificity and values of the kinetics constants (V_{max} and K_m) of PrtL were determined by measuring the proteinase activity at optimal conditions, using different substrate concentrations up to 2000 μM: S-Ala, succinyl-alanyl-glutamyl-prolyl-phenylalanine-*p*-nitroanilide (S-Glu; Bachem) and methoxy-succinyl-arginyl-prolyl-tyrosyl-*p*-nitroanilide (MS-Arg; Kabi Diagnostica). Kinetics constants were calculated by directly fitting data to the Michaelis–Menten equation by nonlinear regression.

Casein hydrolysis

Casein degradation was carried out following the protocol described by Espeche Turbay et al. (2009). Whole cell-bound PrtL and purified PrtL were incubated with 3 mg ml^{-1} of α-, β-, and κ-casein (Sigma), previously dissolved in 100 mM phosphate buffer (pH 7.0), at a ratio of 1:1 (vol vol^{-1}) at 37 °C for 4 h. Samples were taken at 0, 15, 30, and 240 min. When necessary, cells were removed by centrifugation, and the supernatants containing different peptide fractions were stored at −20 °C. Casein hydrolysis was monitored by SDS-PAGE as previously described (Espeche Turbay et al. 2009) and visualized by silver staining (Bio-Rad).

Nucleotide sequencing and sequence analyses

Whole-genome sequencing of *L. delbrueckii* subsp. *lactis* CRL 581 was performed with a 454 GS Titanium pyrosequencer at INDEAR, Argentina (Hebert et al. 2013). The data from this whole-genome shotgun project were deposited in GenBank under accession number ATBQ00000000.

Protein homology searches were carried out with the SWISS-PROT database with the EMBL BLAST and EMBL FASTA servers. PrtL sequence analyses (alignments, comparisons, translations) were completed using DNAman 5.5.2 (Lynnon BioSoft).

Statistical analyses

Statistical analyses were performed with the software package Minitab 14 (Minitab Inc.) using ANOVA general linear models followed by Tukey's post hoc test, and $P<0.05$ was considered significant. Unless otherwise indicated, all values were the means of three independent trials±standard deviation. No significant differences were observed between individual replicates.

Results

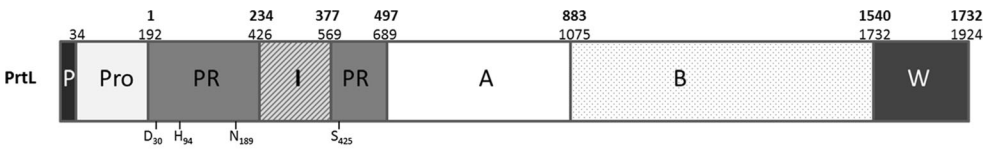
Identification, DNA sequencing, and analysis of PrtL

The genome of *L. delbrueckii* subsp. *lactis* CRL 581 was sequenced and analyzed, showing the presence of only one gene that encodes a cell envelope-associated serine proteinase, named PrtL (Hebert et al. 2013). No sequences homologous to the proteinase maturation protein PrtM were found in the genome of *L. delbrueckii* subsp. *lactis* CRL 581. Analysis of the deduced PrtL sequence showed that this proteinase consists in 1924 amino acids, and a precursor-predicted relative molecular mass of 209,215 Da. PrtL is organized in several structural and/or functional domains characteristic of CEP from LAB (Savijoki et al. 2006; Siezen 1999). This enzyme is synthesized as a preproprotein and becomes mature upon removal of the PPro domain, which consists of a putative 34-amino acid signal sequence and a prosequence of 158 amino acids (Fig. 1), being the residues between Thr^{192} and Asp^{193}, the predicted cleavage site. Therefore, the processed mature PrtL protein would contain 1733 residues with a predicted molecular mass of 188 kDa and an isoelectric point of 6.32. The N-terminal region of the mature PrtL proteinase (497 amino acids) is the catalytic domain (PR), which contains a large insert (I, 144 amino acids). This PR domain has similarity to the subtilisin-like serine proteinases known as subtilases; thus, PrtL can be classified within this family (Siezen 1999). This domain is characterized by a catalytic triad, Asp^{30}, His^{94}, and Ser^{425} (Fig. 1). The subsequent domains are the A domain (386 amino acids), the B domain (657 amino acids), and the W domain (192 amino acids) (Fig. 1). The AN domain described in some proteinases (Siezen 1999), which contains the LPxTG motif and functions as an anchor to the peptidoglycan layer, was not found in PrtL, suggesting that PrtL does not bind to the cell surface via the sortase machinery (Marraffini et al. 2006; Navarre and Schneewind 1994).

PrtL extraction by high ionic strength

To assess the involvement of electrostatic interactions in the binding of PrtL to the bacterial cell envelope, *L. delbrueckii* subsp. *lactis* CRL 581 cells were incubated under different ionic strength conditions and centrifuged, and the proteolytic activity was assayed in the pellet and supernatant fractions (Table 1). After incubating CRL 581 cells with buffer containing 0.5 M NaCl, PrtL remained mainly associated to the cell. However, upon cell treatment with 1 M NaCl, 80 % of PrtL was released in the supernatant (Table 1). A similar PrtL release percentage was obtained with 3 M NaCl treatment (data not shown). These results were corroborated by SDS-PAGE analysis; a band of ~190 kDa (which is close to the mature PrtL-predicted molecular mass) was found mostly in the supernatant fraction after treatments with 1 and 3 M NaCl (Fig. 2). This ~190-kDa protein band was excised from the gel and identified by MALDI-TOF MS-MS analysis as PrtL (gi|511181292), a subtilase family protein derived from *L. delbrueckii* subsp. *lactis* CRL 581. Since increasing salt concentration reduces the strength of ionic binding by providing competing ions for the charged residues (Alberts et al. 2002; Heimburg and Marsh 1995), these results indicated that PrtL binding is primarily controlled by electrostatic forces.

In order to compare the binding mechanism of different CEP of LAB to the cell, we subjected the proteinases from *L. helveticus* CRL 1062 (PrtH), *Lc. lactis* CRL 1195 (PrtP), and *S. thermophilus* CRL 1185 (PrtS) to different ionic strength treatments (Table 1). In the presence of 0.5 M NaCl, approximately 80 % of PrtH activity was found in the supernatant fraction, suggesting that the ionic interaction of



CEP	% of identity				
PrtB	95	87	96	91	93
PrtH	46	30	31	27	24
PrtH2	39	nd	25	15	31
PrtP	45	28	31	26	ns
PrtR	37	nd	28	18	ns
PrtS	34	29	ns	nd	ns

Fig. 1 Schematic representation of the predicted domains of PrtL and multiple sequence alignment percentages of PrtL with different CEP of LAB. Each *number* indicates the last amino acid of a domain; *top numbers* refer to the mature proteinase, and *bottom numbers* indicate the unprocessed enzyme. The catalytic triad and the amino acid number corresponding in the catalytic domain are indicated. *PPro* preprodomain, *PR* catalytic proteinase domain, *I* insert domain, *A* A domain, *B* B domain, *W* cell wall domain. The analysis was performed for PrtL of *L. delbrueckii* subsp. *lactis* CRL 581 (accession number EPB98635), PrtB of *L. delbrueckii* subsp. *bulgaricus* NCDO1489 (accession number L48487), PrtH (accession number AAD50643), and PrtH2 (accession number WP_020829174) of *L. helveticus* CNRZ 32, PrtP of *Lc. lactis* subsp. *cremoris* SK11 (accession number A32364), PrtR of *L. rhamnosus* BGT10 (accession number CAD43138), and PrtS of *S. thermophilus* CNRZ 385 (accession number AAG09771). *nd* domain not found, *ns* not significant (i.e. <25 %)

Table 1 Effect of NaCl on the release of CEP of *L. delbrueckii* subsp. *lactis* CRL 581, *L. helveticus* CRL 1062, *Lc. lactis* CRL 1195, and *S. thermophilus* CRL 1185

Treatment	Proteinase activity (%)[a]							
	L. delbrueckii subsp. *lactis* CRL 581 (PrtL)		*L. helveticus* CRL 1062 (PrtH)		*Lc. lactis* CRL 1195 (PrtP)		*S. thermophilus* CRL 1185 (PrtS)	
	Cell-bound	Released	Cell-bound	Released	Cell-bound	Released	Cell-bound	Released
No NaCl	97.5±3.6	2.5±3.5	92.0±4.2	8.0±4.3	91.7±5.6	8.3±5.1	100	0
0.5 M NaCl	81.9±5.8	18.1±5.8	19.6±8.5	80.4±7.0	81.0±9.9	19.0±6.8	100	0
1 M NaCl	11.8±2.5	88.2±2.5	20.0±3.7	80.0±6.4	83.3±4.1	16.7±4.0	100	0

[a] The values of proteinase activity are expressed as percentages of proteinase activity with regard to untreated cells. Activity values are the means± standard deviations from three independent experiments

PrtH with the cell envelope was weaker than that observed for PrtL. The W domains of PrtL and PrtH are basic with a content of 31.8 and 15.9 % of lysine residues, respectively. On the other hand, PrtP and PrtS, which are anchored to the cell wall by a dedicated sortase LPxTG motif (Marraffini et al. 2006; Navarre and Schneewind 1994; Siezen 1999), were not released from the cells upon salt treatment (Table 1).

Purification and biochemical characterization of PrtL

In order to study the biochemical properties and substrate specificity of *L. delbrueckii* subsp. *lactis* CRL 581 proteinase, the released enzyme after 1 M NaCl treatment was retained by an ultrafiltration membrane with a cutoff of 100 kDa. This step allowed us to remove most of the contaminating proteins, obtaining a partially purified proteinase, as observed by the presence of a band with the expected molecular mass in the SDS-PAGE (Fig. 3, lane 3).

The optimal temperature for the S-Ala hydrolysis by the purified PrtL was observed between 37 and 40 °C, decreasing sharply with temperatures above 45 °C (Fig. 4a). On the other hand, the maximum PrtL activity levels were observed between pH 7.0 and 8.0, decreasing afterwards. The enzyme was relatively stable at pH 6.0, still showing approximately 60 % of the activity observed at pH 7.5. At pH 5.0 and pH 9.0, the enzyme showed about 35 % of the activity presented at optimal pH (Fig. 4b).

In order to gain information about the substrate specificity of the purified PrtL, the kinetics of the enzyme reaction with different *p*-nitroanilide derivatives, S-Ala (a non-charged chromophoric peptide), S-Glu (a negatively charged chromophoric peptide), and MS-Arg (a positively charged chromophoric peptide), was determined (Table 2). Proteolytic activity with S-Ala and S-Glu showed a classical Michaelis and Menten saturation, with no significant difference in the V_{max} values. However, PrtL exhibited higher affinity for S-Ala than

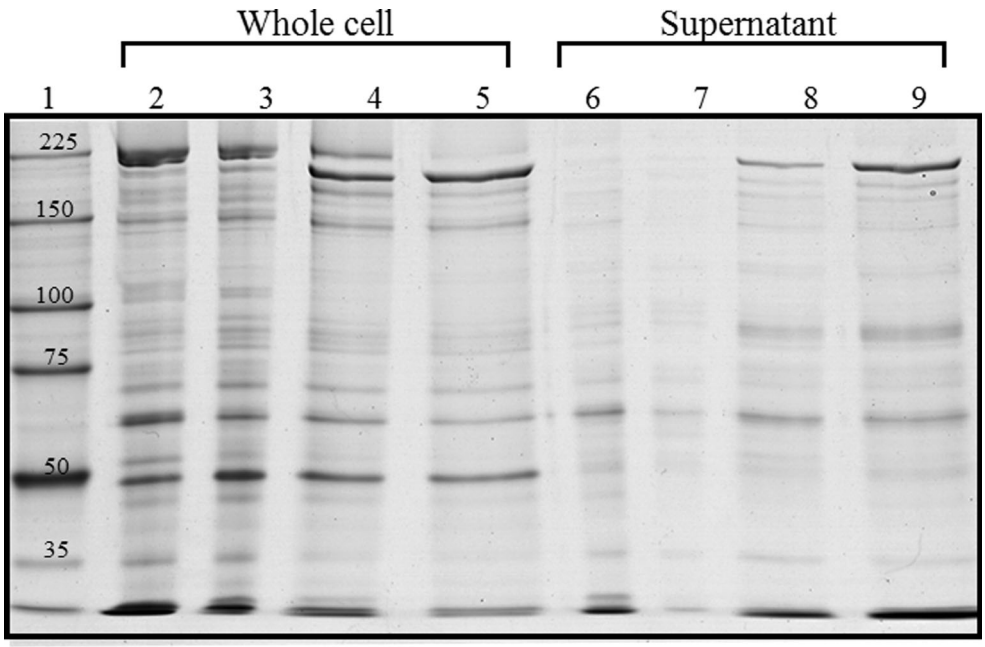


Fig. 2 SDS-PAGE analysis of PrtL of *L. delbrueckii* subsp. *lactis* CRL 581 extracted from the cell under high ionic strength conditions. SDS-PAGE of PrtL whole cells and supernatant fractions obtained after treatment of *L. delbrueckii* subsp. *lactis* CRL 581 with 20 mM Tris–HCl buffer (pH 7.5) in the presence of different concentrations of NaCl. *Lanes 2* and *6* without NaCl, *lanes 3* and *7* incubated with 0.5 M NaCl, *lanes 4* and *8* incubated with 1 M NaCl, *lanes 5* and *9* incubated with 3 M NaCl. *Lane 1* molecular weight marker (kDa; Promega). The 8 % gel was stained with Coomassie Brilliant Blue G-250. Data are representative of three independent experiments

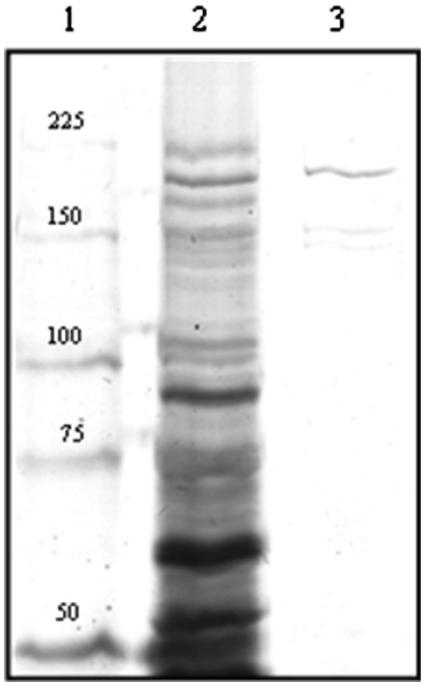


Fig. 3 SDS-PAGE of the purified PrtL of *L. delbrueckii* subsp. *lactis* CRL 581. *Lane 1* molecular weight marker (kDa; Promega), *lane 2* whole cells of *L. delbrueckii* subsp. *lactis* CRL 581, *lane 3* 1 M NaCl supernatant fraction obtained after filtered through a 100-kDa membrane (100,000 MWCO, Amicon, Millipore). Gel was stained with Coomassie Brilliant Blue G-250. Data are representative of three independent experiments

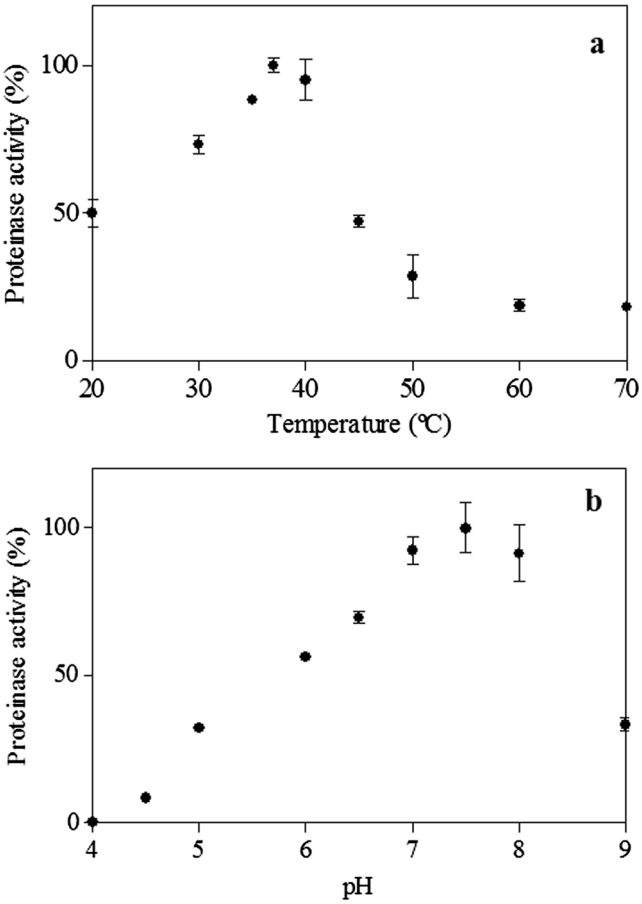


Fig. 4 Thermal and pH profile of PrtL activity of *L. delbrueckii* subsp. *lactis* CRL 581. Proteolytic activity of purified PrtL was measured at different temperatures (**a**) and pH (**b**) using the chromogenic substrate succinyl-alanyl-alanyl-prolyl-phenylalanine-*p*-nitroanilide. In each case, data were normalized by the enzymatic activity at 37 °C and pH 7.5. Data are representative of three independent experiments

for S-Glu as measured by its K_m values (Table 2). On the contrary, no detectable activity was observed with MS-Arg as substrate.

Casein fraction hydrolysis

The caseinolytic specificity of the purified and bound PrtL was also analyzed. The ability of purified PrtL to hydrolyze α-, β-, and κ-casein was tested at different times and compared with those of the cell envelope-bound proteinase (Fig. 5). A similar degradation pattern was observed for the partial purified as well as by the cell-bound proteinase; PrtL hydrolyzed β-casein predominantly and α-casein at a lower rate (Fig. 5). However, the kinetics of hydrolysis was different as the purified proteinase was more efficient in the degradation of β-casein. On the other hand, no κ-casein hydrolysis was observed for the purified PrtL, as previously observed for the cell-bound PrtL (data not shown) (Hebert et al. 2008).

Discussion

The CEP of *L. delbrueckii* subsp. *lactis* CRL581 plays a key role in milk fermentation processes because it enables this strain to grow in milk, thereby ensuring successful fermentation. Furthermore, this CEP contributes to the health-beneficial properties of fermented milk products because it can release bioactive peptides during milk fermentation (Hayes et al. 2007; Hebert et al. 2010; Saavedra et al. 2013; Villegas et al. 2014). Sequence analysis of *L. delbrueckii* subsp. *lactis* CRL 581 genome revealed the presence of only one gene that encodes PrtL (Hebert et al. 2013). In *Lc. lactis*,

Table 2 Kinetics parameters of PrtL in the presence of different substrates

Substrates	V_{max} [a]	K_m [a]
S-Ala	317.9±47	461.7±55
S-Glu	369.2±45	553.5±40
MS-Arg	ND	ND

ND not detected

[a] V_{max} and K_m were expressed as nmol pNA min^{-1} mg $prot^{-1}$ and μM, respectively

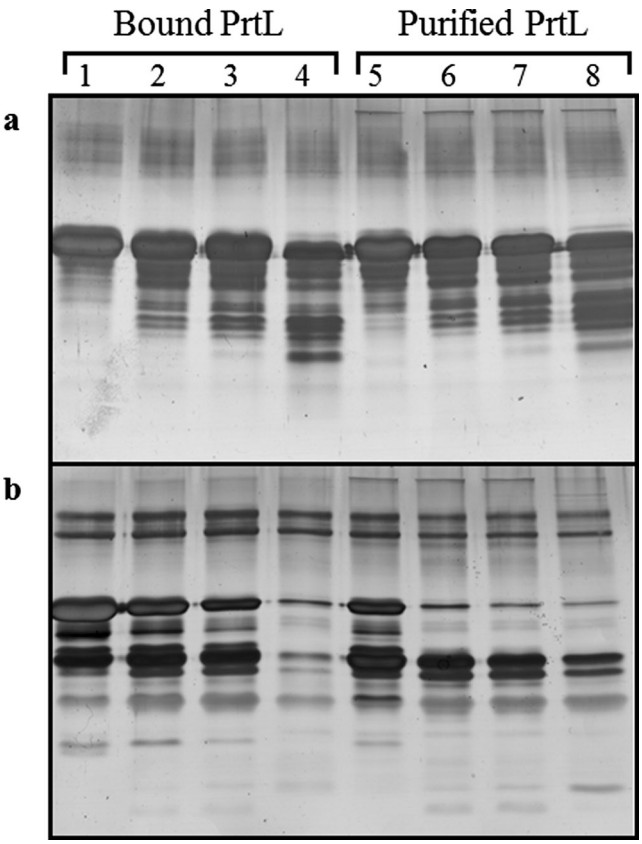


Fig. 5 Caseinolytic profiles of cell-bound and released PrtL of *L. delbrueckii* subsp. *lactis* CRL 581. Hydrolysis of α-casein (**a**) and β-casein (**b**) by the cell envelope-bound and the purified PrtL. Incubation times 0 min (*lanes 1* and *5*), 15 min (*lanes 2* and *6*), 30 min (*lanes 3* and *7*), and 240 min (*lanes 4* and *8*). Data are representative of three independent experiments

Lactobacillus johnsonii, *L. rhamnosus*, *Lactobacillus casei*, and *L. paracasei*, the proteinase gene (*prtP*) is preceded by a divergently transcribed gene encoding a membrane-bound lipoprotein (PrtM) that was shown to be essential for autocatalytic maturation of the proteinase (Haandrikman et al. 1991; Holck and Naes 1992; Liu et al. 2010). However, in *L. delbrueckii* subsp. *lactis* CRL 581, as well as in *L. acidophilus*, *L. helveticus*, *S. thermophilus*, and *L. delbrueckii* subsp. *bulgaricus*, no *prtM* gene was identified in the *prtL* flanking regions (Liu et al. 2010).

The PrtL molecular mass is close to that reported for other described CEP such as PrtP from *L. paracasei* subsp. *paracasei* (Holck and Naes 1992), *Lc. lactis* subsp. *cremoris* Wg2 (Vos et al. 1989), *Lc. lactis* subsp. *lactis* NCDO763 (Kiwaki et al. 1989), *Lc. lactis* subsp. *cremoris* SK11 (Bruinenberg et al. 2000), PrtR from *L. rhamnosus* (Pastar et al. 2006), PrtB from *L. delbrueckii* subsp. *bulgaricus* (Gilbert et al. 1996), and PrtH and PrtH2 from *L. helveticus* CNRZ 32 (Genay et al. 2009).

In most cases, the isolation of CEP from LAB was achieved by repeated treatment of cells with a calcium-free buffer, which induces the loss of weakly bound Ca^{2+} ions, leading to the exposure of a sequence that is highly susceptible to autoproteolytic attack. Previously, we demonstrated that PrtL activity, stability, and release were independent of the presence of Ca^{2+} ions in the medium, and the release of PrtL occurred mainly at pH above 6 (Espeche Turbay et al. 2009). The C-terminal region of PrtL lacks the LPxTG motif (AN domain) necessary for a covalent linkage with the cell envelope. Meanwhile, its W domain contains a high content of lysine (31.8 %), suggesting that PrtL binding to the cell wall occurs through electrostatic interactions with the negatively charged teichoic acids of the cell wall. Here, we demonstrated that PrtL was released from the cell envelope after treatment with 1 M NaCl. Since increasing salt concentration reduces the strength of ionic binding by providing competing ions for the charged residues (Alberts et al. 2002; Heimburg and Marsh 1995), our results confirmed, for the first time, that PrtL binding is primarily controlled by electrostatic forces. Electrostatic interactions between PrtL and the cell wall can also be modulated by changing the protonation/deprotonation state of charged residues via environmental pH. When the pH is below the isoelectric point of PrtL (p*I*=6.32), lysine residues are protonated and carry positive charges, leading to electrostatic interactions with the cell. On the other hand, when the pH is higher than the p*I*, lysine residues tend to be deprotonated, resulting in repulsive interactions between PrtL and the teichoic acids of the cell wall. Therefore, at a working pH of 7, the electrostatic repulsion forces that arise upon deprotonation could be an extra driving force for PrtL releasing (Espeche Turbay et al. 2009).

The CEP extraction method used in this study effectively released LAB proteinases that are non-covalently linked to the cell envelope. Several advantages were observed for the proposed extraction method: (1) it is a mild treatment that did not modify or collapse cellular integrity, (2) it allowed ~90 % of proteinase release, which is highly efficient when compared with the values reported after pH treatment (Espeche Turbay et al. 2009), and (3) it did not compromise the CEP structure since no modification in the proteinase activity or SDS-PAGE band size was detected; proteinase release by incubation of the cells with a calcium-free buffer leads to a truncated proteinase (Bruinenberg et al. 2000; Exterkate and Alting 1999), sometimes with a decrease in specific activity and a dramatic reversible reduction in thermal stability (Exterkate and Alting 1999).

After 1 M NaCl extraction, PrtL was purified and biochemically characterized. Our results differed from the crude proteinase extract from *L. delbrueckii* subsp. *lactis* ACA-DC 178, obtained by Tsakalidou et al. (1999), which showed highest activity at pH 6.0 and 40 °C. It is worth to mention that this proteinase showed different specificity respect to PrtL from *L. delbrueckii* subsp. *lactis* CRL 581 (Hebert et al. 2008; Tsakalidou et al. 1999). In addition, the CEP from *L. delbrueckii* subsp. *lactis* ACA-DC 178 was removed from

Table 3 Substitution of amino acids at positions relevant for substrate binding in PrtL and other CEP of LAB

CEP	Sequence at indicated site[a]								
	Source	131	138	142	144	166	177	222	747	748
PrtL	*L. delbrueckii* subsp. *lactis* CRL 581	Ser	Gly	Asp	Ile	Val	Gln	Ala	Gly	Thr
PrtB	*L. delbrueckii* subsp. *bulgaricus* NCDO1489	Ser	Gly	Asp	Ile	Val	Gln	Thr	Gly	Thr
PrtP	*L. paracasei* NCDO151	Thr	Thr	Ala	Leu	Asp	Leu	Thr	Gln	Thr
PrtP	*Lc. lactis* subsp. *cremoris* Wg2	Thr	Thr	Ser	Leu	Asp	Leu	Thr	Leu	Thr
PrtP	*Lc. lactis* subsp. *cremoris* SK11	Ser	Lys	Ala	Val	Asn	Leu	Ser	Arg	Lys
PrtH	*L. helveticus* CNRZ 32	Ser	Ser	Thr	Ile	Val	Val	Asn	Ala	Thr
PrtH2	*L. helveticus* CNRZ 32	Gly	Asn	Leu	Met	Gly	Gln	Gly	Val	Glu
PrtR	*L. rhamnosus* BGT10	Asp	Asp	Asn	Ile	Gly	Gln	Nd	Thr	Thr
PrtS	*S. thermophilus* CNRZ 385	Ser	Thr	Ala	Tyr	Ala	Thr	Ala	Gly	Asp

[a] Numbering corresponds to the mature PrtP of *Lc. lactis* subsp. *cremoris* SK11

the cell envelope by washing the cells with a calcium-free buffer, denoting that this CEP is distinct than PrtL. On the other hand, PrtB of *L. delbrueckii* subsp. *bulgaricus* CNRZ 397, PrtS of *S. thermophilus* CNRZ 385, and PrtH of *L. helveticus* L89 and *L. helveticus* CRL 1062 showed an optimum temperature of 42, 37, 50, and 42 °C and an optimum pH of 5.5, 7.5, 6.2, and 7.0, respectively (Fernandez-Espla et al. 2000; Hebert et al. 1999; Laloi et al. 1991; Martín-Hernández et al. 1994).

To study the substrate specificity of the purified PrtL, three different *p*-nitroanilide derivatives were used. Among them, the substrates MS-Arg and S-Glu can be used to distinguish between CEP_I and CEP_{III}; the activity of CEP_I is lower, and that of CEP_{III} is higher toward the S-Glu substrate than the respective activities toward MS-Arg (Exterkate et al. 1993). While PrtL presented activity with S-Ala and S-Glu, no detectable activity was observed with MS-Arg, a feature also shared by the proteinase of *L. delbrueckii* subsp. *bulgaricus* CNRZ 397 (PrtB) (Laloi et al. 1991). Protein engineering studies employing hybrid Wg2 (CEP_I)–SK11 (CEP_{III}) proteinases of *Lc. lactis* identified the N-terminal fragment of the mature proteinase (particularly positions 131, 138, 142, 144, 166, 177, and 222 of the *Lc. lactis* subsp. *cremoris* SK11 PrtP) as well as the large C-terminal containing residues 747 to 748 as sites that significantly affect casein cleavage specificity (Table 3) (Kunji et al. 1996; Vos et al. 1991). These substrate binding regions are different among PrtL, PrtB, PrtH, PrtP, PrtR, and PrtS (Table 3). PrtL has a unique amino acid substitution at position 222 (Ala) compared to PrtB (Table 3). The highest sequence identity among the CEP from LAB is within the N-terminal catalytic domain, showing PrtL an identity of about 95, 46, and 45 % with PrtB, PrtH, and PrtP, respectively (Fig. 1). Unlike PrtB, PrtL does not show the involvement of cysteine residues in catalysis. These data, together with the specificity of PrtL towards the different chromophoric peptides and the unique combination of amino acids at residues thought to be involved in substrate specificity, indicate that this enzyme does not fit into any of the groups distinguished so far among the lactococcal CEP variants (Exterkate et al. 1993) and can be classified as a new group of CEP. On the other hand, a similar degradation pattern was observed for the purified as well as by the cell-bound proteinase. These results suggested that the released proteinase did not change its conformation since no modification in the stability and caseinolytic activity was observed.

The data presented in this study contribute to enlarge the limited knowledge on thermophilic lactobacilli CEP; PrtL was extracted from the cell under high ionic strength conditions, purified, and biochemically characterized. The use of 1 M NaCl did not affect the stability, activity, and substrate specificity of PrtL. These results are important from an industrial point of view since during the manufacture of hard cheeses, high concentrations of NaCl are present, and PrtL would remain active either bound to the cell or released, maintaining the beneficial health effects of the fermented milk products. The proteolysis reactions are quite complex; thus, it is necessary to study extensively the proteinase mechanism to control, for instance, the qualities in industrial and biotechnological products (Nielsen 2002). In addition, the broad knowledge about mechanism of anchoring proteins to the cell envelope and the development of efficient and commercially viable methods for CEP extraction will open new possibilities of their applications.

Acknowledgments This work was supported by CONICET, ANPCyT, MINCyT, and FONARSEC from Argentina.

References

Alberts B, Johnson A, Lewis J, Raff M, Roberts K, Walter P (2002) Molecular biology of the cell, 4th edn. Garland Science, New York

Bruinenberg PG, De Vos WM, Siezen RJ (2000) Deletion of various carboxy-terminal domains of *Lactococcus lactis* SK11 proteinase: effects on activity, specificity, and stability of the truncated enzyme. Appl Environ Microbiol 66:2859–2865

Callanan M, Kaleta P, O'Callaghan J, O'Sullivan O, Jordan K, McAuliffe O, Sangrador-Vegas A, Slattery L, Fitzgerald GF, Beresford T, Ross RP (2008) Genome sequence of *Lactobacillus helveticus*, an organism distinguished by selective gene loss and insertion sequence element expansion. J Bacteriol 190:727–735

Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG (2004) Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25:1327–1333

Espeche Turbay MB, Savoy de Giori G, Hebert EM (2009) Release of the cell-envelope-associated proteinase of *Lactobacillus delbrueckii* subspecies *lactis* CRL 581 is dependent upon pH and temperature. J Agric Food Chem 57:8607–8611

Espeche Turbay MB, de LeBlanc De Moreno A, Perdigon G, de Savoy Giori G, Hebert EM (2012) β-Casein hydrolysate generated by the cell envelope-associated proteinase of *Lactobacillus delbrueckii* ssp. *lactis* CRL 581 protects against trinitrobenzene sulfonic acid-induced colitis in mice. J Dairy Sci 95:1108–1118

Exterkate FA (1995) The lactococcal cell envelope proteinases: differences, calcium-binding effects and role in cheese ripening. Int Dairy J 5:995–1018

Exterkate FA, Alting AC (1999) Role of calcium in activity and stability of the *Lactococcus lactis* cell envelope proteinase. Appl Environ Microbiol 65:1390–1396

Exterkate FA, Alting AC, Bruinenberg PG (1993) Diversity of cell envelope proteinase specificity among strains of *Lactococcus lactis* and its relationship to charge characteristics of the substrate-binding region. Appl Environ Microbiol 59:3640–3647

Fernandez-Espla MD, Garault P, Monnet V, Rul F (2000) *Streptococcus thermophilus* cell wall-anchored proteinase: release, purification, and biochemical and genetic characterization. Appl Environ Microbiol 66:4772–4778

Genay M, Sadat L, Gagnaire V, Lortal S (2009) *prtH2*, not *prtH*, is the ubiquitous cell wall proteinase gene in *Lactobacillus helveticus*. Appl Environ Microbiol 75:3238–3249

Gilbert C, Atlan D, Blanc B, Portalier R, Germond JE, Lapierre L, Mollet B (1996) A new cell surface proteinase: sequencing and analysis of the *prtB* gene from *Lactobacillus delbrueckii* subsp. *bulgaricus*. J Bacteriol 178:3059–3065

Haandrikman AJ, Kok J, Venema G (1991) The lactococcal proteinase-maturation protein prtM is a lipoprotein. J Bacteriol 173:4517–4525

Hayes M, Ross RP, Fitzgerald GF, Stanton C (2007) Putting microbes to work: dairy fermentation, cell factories and bioactive peptides. Part I: overview. Biotechnol J 2:426–434

Hebert EM, Raya RR, De Giori GS (1999) Characterisation of a cell-envelope proteinase of *Lactobacillus helveticus*. Biotechnol Lett 21: 831–834

Hebert EM, Raya RR, de Giori GS (2002) Modulation of the cell-surface proteinase activity of thermophilic lactobacilli by the peptide supply. Curr Microbiol 45:385–389

Hebert EM, Raya RR, de Giori GS (2004) Nutritional requirements of *Lactobacillus delbrueckii* subsp. *lactis* in a chemically defined medium. Curr Microbiol 49:341–345

Hebert EM, Mamone G, Picariello G, Raya RR, Savoy G, Ferranti P, Addeo F (2008) Characterization of the pattern of as1- and b-casein breakdown and release of a bioactive peptide by a cell envelope proteinase from *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. Appl Environ Microbiol 74:3682–3689

Hebert EM, Saavedra L, Ferranti P (2010) Bioactive peptides derived from casein and whey proteins. In: Mozzi F, Raya R, Vignolo G (eds) Biotechnology of lactic acid bacteria: novel applications. Wiley-Blackwell, Ames, Iowa, USA, pp 233–249

Hebert EM, Raya RR, Brown L, Font de Valdez G, Savoy de Giori G, Taranto MP (2013) Genome sequence of the cheese-starter strain *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. Genome Announcements 1

Heimburg T, Marsh D (1995) Protein surface-distribution and protein-protein interactions in the binding of peripheral proteins to charged lipid membranes. Biophys J 68:536–546

Holck A, Naes H (1992) Cloning, sequencing and expression of the gene encoding the cell-envelope-associated proteinase from *Lactobacillus paracasei* subsp. *paracasei* NCDO151. J Gen Microbiol 138:1353–1364

Jarocki P, Podlesny M, Wasko A, Siuda A, Targonski Z (2010) Differentiation of three *Lactobacillus rhamnosus* strains (E/N, Oxy and Pen) by SDS-PAGE and two-dimensional electrophoresis of surface-associated proteins. J Microbiol Biotechnol 20:558–562

Kiwaki M, Ikemura H, Shimizu-Kadota M, Hirashima A (1989) Molecular characterization of a cell wall-associated proteinase gene from *Streptococcus lactis* NCDO763. Mol Microbiol 3: 359–369

Kunji ER, Mierau I, Hagting A, Poolman B, Konings WN (1996) The proteolytic systems of lactic acid bacteria. Antonie Van Leeuwenhoek 70:187–221

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Laloi P, Atlan D, Blanc B, Gilbert C, Portalier R (1991) Cell-wall-associated proteinase of *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397: differential extraction, purification and properties of the enzyme. Appl Microbiol Biotechnol 36:196–204

Liu M, Bayjanov JR, Renckens B, Nauta A, Siezen RJ (2010) The proteolytic system of lactic acid bacteria revisited: a genomic comparison. BMC Genomics 11:36–51

Marraffini LA, Dedent AC, Schneewind O (2006) Sortases and the art of anchoring proteins to the envelopes of gram-positive bacteria. Microbiol Mol Biol Rev 70:192–221

Martín-Hernández MC, Alting AC, Exterkate FA (1994) Purification and characterization of the mature, membrane-associated cell-envelope proteinase of *Lactobacillus helveticus* L89. Appl Microbiol Biotechnol 40:828–834

Navarre WW, Schneewind O (1994) Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in gram-positive bacteria. Mol Microbiol 14:115–121

Nielsen SS (2002) Plasmin system and microbial proteases in milk: characteristics, roles, and relationship. J Agric Food Chem 50: 6628–6634

Pastar I, Fira D, Strahinic I, Krstic K, Begovic J, Topisirovic L, Jovanovic G (2006) Analysis of the presence of *prtR* proteinase gene in natural isolates of *Lactobacillus rhamnosus*. Folia Microbiol (Praha) 51:535–540

Pederson JA, Mileski GJ, Weimer BC, Steele JL (1999) Genetic characterization of a cell envelope-associated proteinase from *Lactobacillus helveticus* CNRZ32. J Bacteriol 181: 4592–4597

Pescuma M, Espeche Turbay MB, Mozzi F, Font de Valdez G, Savoy de Giori G, Hebert EM (2013) Diversity in proteinase specificity of thermophilic lactobacilli as revealed by hydrolysis of dairy and vegetable proteins. Appl Microbiol Biotechnol 97:7831–7844

Saavedra L, Hebert EM, Minahk C, Ferranti P (2013) An overview of "omic" analytical methods applied in bioactive peptide studies. Food Res Int 54:925–934

Savijoki K, Ingmer H, Varmanen P (2006) Proteolytic systems of lactic acid bacteria. Appl Microbiol Biotechnol 71:394–406

Scolari G, Vescovo M, Zacconi C, Vescovi F (2006) Extraction and partial characterization of proteolytic activities from the cell surface of *Lactobacillus helveticus* Zuc2. J Dairy Sci 89:3800–3809

Siezen RJ (1999) Multi-domain, cell-envelope proteinases of lactic acid bacteria. Antonie Van Leeuwenhoek 76:139–155

Stefanitsi D, Sakellaris G, Garel J-R (1995) The presence of two proteinases associated with the cell wall of *Lactobacillus bulgaricus*. FEMS Microbiol Lett 128:53–58

Thomas TD (1975) Tagatose-1,6-diphosphate activation of lactate dehydrogenase from *Streptococcus cremoris*. Biochem Biophys Res Commun 63:1035–1042

Tsakalidou E, Anastasiou R, Vandenberghe I, Van Beeumen J, Kalantzopoulos G (1999) Cell-wall-bound proteinase of *Lactobacillus delbrueckii* subsp. *lactis* ACA-DC 178: characterization and specificity for β-casein. Appl Environ Microbiol 65:2035–2040

van de Guchte M, Penaud S, Grimaldi C, Barbe V, Bryson K, Nicolas P, Robert C, Oztas S, Mangenot S, Couloux A, Loux V, Dervyn R, Bossy R, Bolotin A, Batto JM, Walunas T, Gibrat JF, Bessieres P, Weissenbach J, Ehrlich SD, Maguin E (2006) The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. Proc Natl Acad Sci U S A 103:9274–9279

Villegas JM, Volentini SI, Rintoul MR, Rapisarda VA (2011) Amphipathic C-terminal region of *Escherichia coli* NADH dehydrogenase-2 mediates membrane localization. Arch Biochem Biophys 505:155–159

Villegas JM, Picariello G, Mamone G, Espeche Turbay MB, Savoy de Giori G, Hebert EM (2014) Milk-derived angiotensin-I-converting enzyme inhibitory peptides generated by *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. Peptidomics 1:22–29

Vos P, Simons G, Siezen RJ, de Vos WM (1989) Primary structure and organization of the gene for a prokaryotic, cell-envelope-located serine proteinase. J Biol Chem 171:2795–2802

Vos P, Boerrigter IJ, Buist G, Haandrikman AJ, Nijhuis M, De Reuver MB, Siezen RJ, Venema G, De Vos WM, Kok J (1991) Engineering of the *Lactococcus lactis* serine proteinase by construction of hybrid enzymes. Protein Eng 4:479–484