

Characterization of new strain *Lactobacillus paracasei* I-N-10 with proteolytic activity: Potential role in decrease in β -casein immuno-reactivity

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Abstract The proteolytic activity of thirty-three LAB isolates from Mongolian tarag was tested on skimmed milk. The strain displaying the highest proteolytic activity was purified and presented by 16S rDNA sequencing 99.9 % homology with *Lactobacillus paracasei* 1-4-2A. It was named *L. paracasei* I-N-10. Proteases of *L. paracasei* I-N-10 hydrolyze predominately β -casein and in some level α_{S2} -casein; hydrolysis of α_{S1} -casein was not observed. Proteolytic activity was optimal at 42 °C and neutral pH. Proteases of *L. paracasei* I-N-10 were inhibited by serine- and metalloproteases inhibitors. PCR amplification revealed the presence of *prtP* gene, which was identical to *prtP* gene of *L. paracasei* genus. Mass spectrometry analysis of β -casein hydrolysate allowed to characterize 7 peptides resulting from proteolysis by *L. paracasei* I-N-10. The isolated strain was able to cleave β -casein in different

sites including 2 of the major linear epitopes implicated in its allergenicity. Being sensitive to main antibiotics classes, *L. paracasei* I-N-10 could be considered as safe and used as starter culture with a potential role in decreasing β -casein immuno-reactivity.

Keywords *Lactobacillus paracasei* · Proteolytic activity · Milk allergy

Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of Gram-positive, low GC content, non-motile bacteria characterized by their capability to ferment sugars to lactic acid. Hence, a variety of LAB, notably *Lactococcus* and *Lactobacillus* spp., because of their unique metabolic characteristics are involved in many fermentation processes of milk, meats, cereals, and vegetables [1] and are used as starter cultures for the production of fermented foods [2]. Lactobacilli are beneficial microorganisms of particular interest because of their long history of use [3]. LAB can be used to produce bulk and fine chemicals, including lactic acid [4], polyols [5], and B vitamins [6]. They are also well known as producers of exopolysaccharides [7, 8], antimicrobial compounds [9], and proteolytic enzymes [10].

Fermentation ensures not only increased shelf life and microbiological safety of a food but also may make some foods more digestible with desirable and pleasant organoleptic characteristics [11].

LAB are usually isolated from the nutrient-rich niches such as plants, fermented foods, and gastrointestinal tract [12]. However, genomic sequencing revealed that most LAB lack biosynthetic pathways for essential amino acids,

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nucleotides, and vitamins [13, 14]. The proteolytic system of LAB is essential for their growth in milk and milk products [15]; it helps them to take up nutrients from milk and contributes significantly to flavor development in fermented milk products.

Milk protein and mainly caseins can be used by many LAB as nitrogen source. Proteases cleave milk proteins into peptides, peptidases cleave the peptides into smaller peptides and amino acids, and thereby antigenic epitopes responsible of milk allergy may be split [16]. As caseins are the main milk allergens, many works focused on and confirm that immuno-reactivity of milk can be decreased by lactobacilli proteolysis [17–19].

Although the beneficial activities of these microorganisms are widely recognized, their efficacy was threatened by resistance to antimicrobials. In recent years, a general belief appeared that additives cultures, probiotics, and starters might act as potential vehicles of antibiotics resistance genes. Consequently, it is important to ascertain that LAB strains, which are going to be used in food, must be free of such genes that could be transferred [20, 21].

Tarag is the local name of Mongolian traditionally homemade yoghurt made from cow, yak, goat, or camel milk. Nomadic populations of Mongolia have acquired technologies for different dairy products (Tarag, koumiss) preparation adapted to their living conditions and climate. Most of these products are exclusively endemic to the country of origin. This sour-tasting beverage was fermented by a co-culture of yeasts and LAB as *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. helveticus*, *Streptococcus thermophilus* [22].

This study describes the characterization of a new *Lactobacillus* strain, *Lactobacillus paracasei* I-N-10, isolated from Mongolian tarag on the basis of its proteolytic activity on different milk protein fractions. The effects of pH, temperature, and the presence of inhibitors on proteolytic activity were also studied. The PCR amplification of proteinase coding genes was performed using the primers specific for different *Lactobacillus* proteinases. Phenotypic strain susceptibility to antibiotics was checked. Moreover, the hydrolysis products were characterized by LC/MS/MS to observe eventual epitopes cleavage.

Materials and methods

Microbiological analyses and cultures conditions

A sample (~10 g) of tarag was aseptically collected, diluted 1/10 (w/v) with sterile 2 % (w/v) trisodium citrate, and homogenized. Serial dilutions were prepared as required, using saline solution (0.9 % NaCl, w/v) and plated on MRS agar (1.5 %, w/v). After 48-h incubation at

different temperatures (30, 37, and 42 °C), colonies produced on the lawn of agar media were transferred to MRS and M17 broth. After purification by streaking on agar media, colonies were analyzed by standard microbiological methods, like microscopic examination, Gram staining, and catalase production. The isolates were routinely propagated in MRS broth medium at 30 °C for 24 h, before carrying out the enzyme assays. For extended periods of storage, they were kept at –20 °C in reconstituted skim milk (10 %; w/v) containing 20 % glycerol (w/v).

Genotypic identification of LAB strains by 16S rDNA sequencing

The molecular identification of active strains was performed by 16S rDNA sequencing. The total DNA of LAB strains was isolated using the method described by Sambrook and Russel [23]. More than 1,500 bp of the 16S rDNA were amplified using universal primer sets: fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5' TAA GGAGGTGATCCAGGC3') [24]. The remaining PCR product of each sample was purified for 16S rDNA sequencing by a DNA purification kit (Bio 101 Inc., Vista, CA, USA). The sequencing was carried out by MilleGen sequencing service (Labège, France). The 16S rDNA sequences of isolates were compared with those available from the GenBank databases using the gapped BLAST program accessed through the National Center for Biotechnology Information server (<http://www.ncbi.nlm.nih.gov>).

Identification of bacteria to the species level was defined as a 16S rDNA sequence similarity of ≥99 % to that of the prototype strain sequence in GenBank.

Proteolytic activity assay

LAB were picked from each pure culture plate and grown at 30 °C in appropriate broth (MRS for lactobacilli and M17 for enterococci and lactococci) until the late exponential growth phase (OD 600 nm, 2.00) was reached. Firstly, a screening of proteolytic active strains was done in UHT skim milk system; strains were reactivated by inoculation (5 % inoculation, v/v) of the previously prepared preculture into UHT skim milk and incubated overnight at 37 °C. Controls were prepared by inoculation of equivalent volume of MRS or M17 media in UHT skim milk. After 24-h incubation, samples of fermented milk were diluted tenfold with sample loading buffer and analyzed by SDS-PAGE. Secondly, for selected strains, proteolytic enzymes activities were determined in non-proliferative cells system. The induction was obtained using milk citrate agar media (MCA): 4.4 % reconstituted skim milk, 0.8 % Na citrate (trisalt), 0.1 % yeast extract, 0.5 % glucose, 1.5 % agar (w/v) [15, 25, 26]. An amount of fresh culture was

spread on the top of the media plate, prior to incubation for 48 h at 37 °C. Over incubation period, cells were collected and washed twice with 0.8 % (w/v) saline solution containing 5 mM CaCl₂ and the pellets were re-suspended to a final OD 600 nm of approximately 10 in 100 mM sodium phosphate (pH 7.2). Sodium caseinate (12 mg/ml), β -casein fraction (4 mg/ml), and denatured whey proteins (5 mg/ml, heated at 85 °C for 20 min) were used as substrates [25, 26]. Each cell suspension was incubated with one substrate at a ratio of 1:1. The resulting mixtures were incubated at 37 °C for 3, 6, 9 or 24 h. Equivalent substrate solutions incubated for the same periods without adding cells were used as controls. The samples were then centrifuged (10,000g, 8 min, 4 °C), and the supernatants were analyzed by SDS-PAGE and reversed-phase high-performance liquid chromatography (RP-HPLC).

Protein profile

SDS-PAGE was performed using the buffer system described by Laemmli [27] and a running acrylamide gel in a Mini PROTEAN 3 system (Biorad, Hercules, CA, USA) gel apparatus. Aliquots of the supernatant as well as controls were combined with sample buffer containing 50 mM Tris-HCl, 10 % (v/v) glycerol, 20 % (w/v) SDS, 5 % (w/v) 2-mercaptoethanol, and 5 % (w/v) Bromophenol blue at pH 6.80. The samples were heated at 100 °C for 5 min and used to load 12 % (w/v) 0.75-mm-thick gels. The gels were run at constant current, 10 mA (for 10 min) in the stacking gel and 20 mA (for 2 h) in the running gel. After electrophoresis, gels were stained with a solution containing 0.05 % Coomassie Brilliant Blue G 250 in 50 % methanol and 10 % acetic acid [28]. Distaining was performed by shaking the gels for 6 h in 7.5 % acetic acid/methanol, and then for 1 h in H₂O.

Whey proteins degradation was measured by Tricine SDS-PAGE on 17 % (w/v) polyacrylamide gels [29]. Gels images were digitized with Image Scanner III (GE Healthcare, USA), and the intensity of the bands was quantified by Image software.

Effects of pH and temperature on proteolytic activity

To investigate the influence of the pH of the medium on the proteolytic activity of the active strains, the method described above was used with some modifications. The cells collected were re-suspended in 100 mM sodium phosphate with adjusted pH values (5.8, 6.2, 6.6, and 7.0) or in 100 mM Tris-HCl buffer (pH 8.0) then mixed with equal amount of substrate and incubated for 24 h at 37 °C. Protein hydrolysis and peptides formation were analyzed by SDS-PAGE. To determine the effect of temperature, a mixture of substrate and cell suspension was maintained for 24 h at

different temperatures (20, 30, 37, and 42 °C). Controls were prepared in the same conditions but without cells.

Effects of proteases inhibitors

Cells were incubated for 1 h at 37 °C in the presence of various inhibitors at a final concentration of 10 mM, prior to adding equal volume of sodium caseinate (12 mg/ml). The inhibitors used were EDTA, iodoacetamide, and Pefabloc. The reaction was stopped after 5-h incubation at 37 °C; then, samples were centrifuged (10,000g, 5 min) and the protein hydrolysis level was determined by SDS-PAGE analysis of supernatants as described above. Substrate solutions without cells and substrate–cells mixture without inhibitor were used as controls.

RP-HPLC

Prior to analysis by RP-HPLC, supernatants obtained after incubation of cells with substrates in non-proliferative cells system were mixed 1/1 (v/v) with 6 M urea and centrifuged (12,000g, 5 min).

Separation of peptides was performed on a Waters HPLC system (Alliance system, Milford, MA, USA) using a Symmetry C₁₈ column (5 μ m, 2.1 mm $\times$ 150 mm) and a Waters 996 photodiode array detector at 220 nm. Two solvents were used: solvent A was 0.055 % (v/v) trifluoroacetic acid (TFA) in Milli-Q water, while solvent B consisted of TFA/HPLC-grade acetonitrile/Milli-Q water, 0.045/80/20 (v/v/v). The gradient was formed by increasing the proportion of solvent B from 0 to 100 % during the first 45 min. The flow rate was set at 0.2 ml/min. Data were analyzed by Millennium 32 Software (Waters).

Mass spectrometry analysis

Mass spectrometry analysis was conducted on the platform “Biopolymers-Interaction-Structural Biology” located at the INRA centre of Nantes (INRA Research Unit 1268) (http://www.nantes.inra.fr/plateformes_et_plateaux_techniques/plateforme_bibs). Peptides were enriched and separated using a Finnigan LCQ ion trap spectrophotometer (Finnigan MAT, San Jose, CA). For peptide identification, MS/MS peak lists were extracted and compared with the protein database using the MASCOT Daemon (version 2.1.3; Matrix Science, London, UK) search engine. The scan rate for MS mode was set between the masses of 600 and 2000 Da. The searches were performed without cleavage enzyme specificity and with variable modifications for oxidation of methionine. MS/MS spectra were searched with a mass tolerance of 1.5 Da for precursor ions and 0.6 for MS/MS fragments. Identification was performed by comparing the obtained peptides with β -casein sequence.

Screening of proteinases genes

Primers were used for proteinases genes detection employing as a template total DNA isolated from *L. paracasei* I-N-10 (Table 1). *Lactobacillus*-specific primers: TI2 and XbA (Tm 55 °C) were designed to target the catalytic domain of *prtR* encoded by *L. rhamnosus* T10 *prtR* gene sequence; P23 and P25 (Tm 55 °C) were designed to recognize the active site residues of *L. helveticus* CNRZ32 *prtH* gene sequence; B10 and B20 (Tm 55 °C) were designed on the basis of *L. delbrueckii* ssp. *bulgaricus* NCDO 1489 *prtB*, and finally the sets M70 and P70 (Tm 56 °C) were prepared to amplify the intergenic region of *PrtP/PrtM* genes and designed according to the *prtP* gene sequence of *Lactococcus lactis* ssp. *cremoris* Wg2. DNA amplifications were done singly with a DNA thermal cycler model (Techno, Barloworld scientific, Cambridge, UK) by using 1 U of Go-Taq polymerase (Promega, Madison WI, USA). The PCR conditions were an initial step of 94 °C for 1 min, followed by amplification using the following thermal profile: 30 cycles of 94 °C for 30 s, annealing at an appropriate temperature depending on Tm of primers for 30 s, and 72 °C for 30 s. Finally, the mixture was heated at 72 °C for 5 min and subsequently cooled to 4 °C until use. Generally, each reaction was analyzed by electrophoresis on 1 % agarose gel.

Assessment of resistance to antibiotics

Susceptibility of *L. paracasei* I-N-10 to seven antibiotics from different classes was tested using a disc diffusion

method on Mueller–Hinton Agar (MHA). The minimal inhibitory concentration (MIC, mg/ml) was determined for all antibiotics: ampicillin, penicillin, gentamicin, kanamycin, tetracycline, chloramphenicol, and vancomycin with concentration ranging from 0.1 to 512 µg/ml. The MIC breakpoint values described by EFSA antimicrobial susceptibility testing standards [30] for *Lactobacilli* were consulted in order to classify *L. paracasei* I-N-10 as sensitive, intermediate, or resistant toward these antibiotics.

Results and discussion

Isolation of proteolytic strains from Tarag

One Mongolian tarag sample used as inoculum produced 33 colonies (25 cocci and 8 bacilli) on MRS and M17 media. The bacterial growth on agar plates was checked for different temperatures with an optimal at 37 °C. The morphological examination and Gram staining showed all isolates were Gram-positive rods and cocci. Isolates were first inoculated in UHT skim milk for 24 h at 37 °C to evaluate their proteolytic activity. SDS-PAGE profile showed that 8 isolates (5 cocci and 3 bacilli) were able to hydrolyze milk proteins. The proteolysis extent after 24-h incubation ranged from 35 to 60 % (data not shown). The isolates were then analyzed for caseins and whey proteins hydrolysis activity using MCA for proteinase activity induction. The same isolates were found to hydrolyze only caseins, but they differed in their efficiency and specificity of proteolysis (data not shown). Therefore, the highest proteolytic isolate was selected, identified by 16S rDNA amplification, and revealed 100 % homology with *Lactobacillus paracasei* 1-4-2A. It was then named *Lactobacillus paracasei* I-N-10.

Proteolytic activity in non-proliferative cells system

As shown in Fig. 1a, hydrolysis of casein started weakly after 3-h incubation and was maximum after 24 h. Proteolytic activity of *L. paracasei* I-N-10 differed according to the casein fraction, β -casein being more efficiently hydrolyzed (100 % hydrolysis after 6 h) than α_{S2} -casein (90 % hydrolysis after 24 h), while hydrolysis of α_{S1} -casein was not significant. SDS-PAGE of the cell-free supernatant generated in the presence of only β -casein (4 mg/ml) was analyzed, showing that the proteolysis started after 3 h of incubation with a near complete hydrolysis of β -casein after 24 h (Fig. 1b).

It is reported that cell-wall bound proteinases are key enzymes for catalyzing the first step in casein degradation [31]. Most experimental data were obtained with lactococcal enzymes, and their proteinases were classified in two groups according to their substrate specificity. PI-type

Table 1 Primers used for the detection of proteinase genes

Target genes	Primers (5'–3')	Product size (bp)	Annealing temperature (°C)
<i>prtP/prtM</i> intergenic region	M70: GCA-TGA-ATT-CAA-TGC-ACG-ATA-AAT-GAG	685	56
	P 70: GCT-TGA-ATT-CGT-TGT-CGC-TGC-GGT-TGT		
<i>prtB</i> catalytic domain	B10: GGT-GTT-GCT-CCT-GAT-GCC-CAG-C	597	56
	B20: CCC-CGT-TTA-ACA-ACT-GCA-AGT-T		
<i>prtH</i> catalytic domain	P23: GCT-TGG-ATA-GTA-GCG-TTA-GC	1,034	56
	P25: GGT-GAA-CAA-ACT-GAA-GAC-G		
<i>prtR</i> catalytic domain	TI2: CAA-CAC-CGG-GAC-CAC-GGT-G	1,052	55
	XbA: CTG-ATC-GTG-GAC-GGT-GTT-GC		

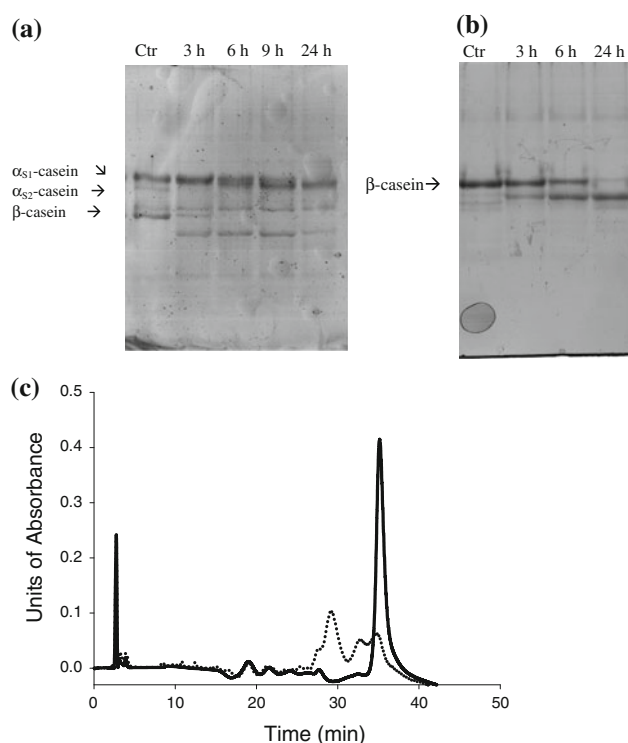


Fig. 1 SDS-PAGE profile of **a** sodium caseinate and **b** β -casein after 3-, 6-, 9-, and 24-h hydrolysis by *Lactobacillus paracasei* I-N-10. Control (Ctr) is sodium caseinate and β -casein in (a) and (b), respectively, without bacterial cells. **c** RP-HPLC profile obtained with *Lactobacillus paracasei* I-N-10 after 24 h of β -casein proteolysis (dot line)

proteinases predominantly hydrolyze β -casein. In contrast, PIII-enzymes degrade all three casein fractions, α_{S1} -, β -, and κ -caseins [32]. As no proteases classification according to their substrates specificity includes α_{S2} -casein and the extremely low activity of *L. paracasei* I-N-10 observed toward α_{S1} -casein, we suggest that *L. paracasei* I-N-10 enzymes are closer to PI-type proteinases.

The hydrolysates were subsequently analyzed by RP-HPLC (Fig. 1c). The chromatograms showed that β -casein digestion by *L. paracasei* I-N-10 gave rise to small hydrophobic peptides eluted essentially between 25 and 30 min (Fig. 1c). The intensity of the peak corresponding to β -casein fraction decreased but was still persisting albeit of small area. The hydrolysis pattern showed the presence of three or four peaks, which may correspond to the bands observed by SDS-PAGE.

Effect of pH on proteases activities

To test the influence of pH conditions on proteolytic activity, the reaction mixtures containing *L. paracasei* I-N-10 cells and sodium caseinate were incubated at various pH values during 24 h at 37 °C. Proteolytic activity was determined starting from pH 5.8 (Fig. 2a). The hydrolysis reached its

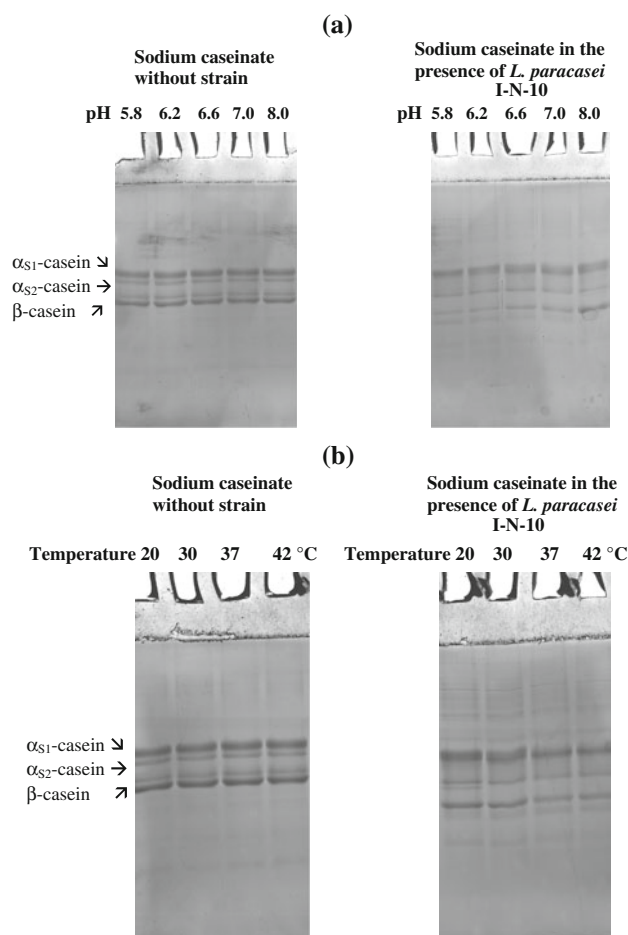


Fig. 2 SDS-PAGE profile of sodium caseinate after 24-h incubation with *Lactobacillus paracasei* I-N-10. Effect of (a) pH (elaborated at 37 °C) and (b) of temperature (elaborated at pH 7.2)

maximum in the pH range 6.6–7.0. The protein profile of generated peptides from caseins hydrolysis was more intense in this interval. The increase in pH to 8.0 did not affect proteolysis. It can be concluded that basic conditions are more favorable for hydrolysis than acidic conditions. Similar observations were previously reported [25, 26, 33].

Effect of temperature on proteases activities

Casein degradation by *L. paracasei* I-N-10 was optimal between 37 and 42 °C (at pH 7.2) (Fig. 2b). At 30 °C, the proteolytic activity was less efficient. In a study of Tsakalidou et al. [34], the optimal temperature for β -casein hydrolysis by *Lactobacillus delbrueckii* subsp. *lactis* ACA-DC178 strain was found to be 40 °C.

Effect of proteases inhibitors

Proteolytic activity of *L. paracasei* I-N-10 was completely inhibited in the presence of EDTA or Pefabloc, inhibitors

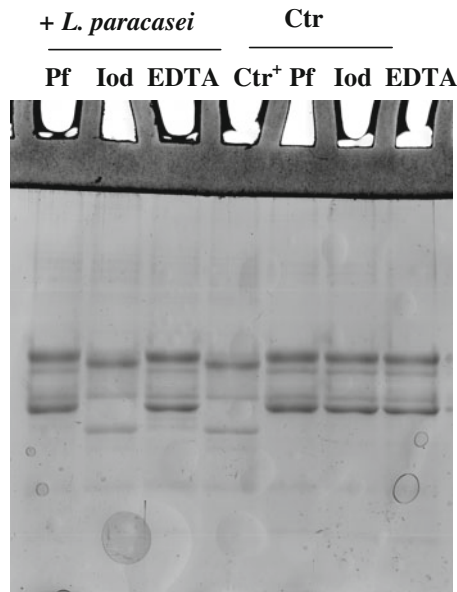


Fig. 3 Effect of protease inhibitors on the proteolytic activity of *Lactobacillus paracasei* I-N-10. Sodium caseinate was used as substrate. *Pf* pefabloc, *Iod* iodoacetic acid, *EDTA* ethylenediaminetetraacetic acid, *Ctr*⁺ sodium caseinate treated by *Lactobacillus paracasei* I-N-10 cells in the absence of inhibitors

of metallo- and serine proteases, respectively (Fig. 3). No inhibition was detected in the presence of iodoacetic acid, a cysteine proteinases inhibitor.

It can be concluded that two types of proteases were involved in the proteolytic activity of *L. paracasei* I-N-10. Several studies yet described the presence of at least two types of proteinases in Lactobacilli [19, 35, 36].

Detection of the proteases genes

The DNA of *L. paracasei* I-N-10 was screened for the presence of known genes of proteases by using specific primers for different types of cell-enveloped proteinases as described by Strahinic et al. [37].

The specific primer for the *prot P*/M gives a PCR fragment of the expected size (685 pb) (Fig. 4), indicating the presence of *prot P* gene in *L. paracasei* I-N-10. The protease P has been identified in *L. paracasei* ssp. *paracasei* BGHN14 [38] then cloned, sequenced from *L. paracasei* NCDO 151 [39]. To date, all results published confirm that all proteinase-positive strains of *L. paracasei* should carry the *prot P* gene associated with the upstream located and divergently transcribed *prot M* gene responsible for the maturation of the protease P [31].

Although the *prt P* gene shows more than 95 % sequence identity with lactococci, those described in *L. paracasei* are chromosomally located [38, 40] despite a plasmid location in lactococci was described [41].

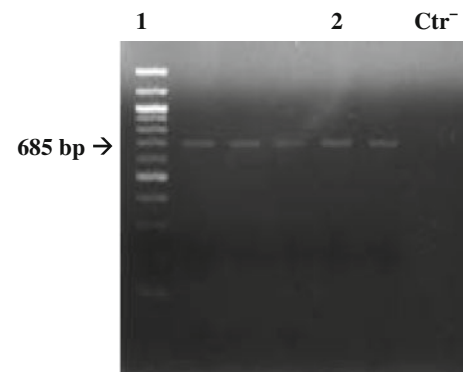


Fig. 4 PCR amplification of *prot P* gene in *Lactobacillus paracasei* I-N-10. Agarose gel 1.2 %, *Ctr*⁻, PCR mix without bacterial DNA; lane 1, 100-bp DNA ladder; lane 2, amplification product obtained with P70/M70 primer sets by *Lactobacillus paracasei* I-N-10

Table 2 Antibiotic resistance profile of *Lactobacillus paracasei* I-N-10

Antibiotics	MIC breakpoints (mg/ml)	Antibiotic susceptibility	Status
Ampicillin	2	1–2	S
Penicillin	4	4	S
Tetracycline	4	4	S
Gentamicin	32	16	S
Chloramphenicol	4	8	R
Vancomycin	nr	nr	nr

R resistant, *S* sensitive, *nr* not required

Antibiotic resistance

Phenotypic antibiotic resistance was investigated by MIC analysis using the microbiological breakpoint level for *L. paracasei* to distinguish between susceptible and resistant strains. *L. paracasei* I-N-10 displaying MIC values lower than breakpoint for ampicillin (MIC ≤ 2) and for penicillin (MIC ≤ 4) was considered as a sensitive strain (Table 2). Similar results were obtained [42] for LAB isolated from Galician raw milk cheeses, which are susceptible to β-lactams (ampicillin and penicillin). Several previous studies showed that LAB from food are quite sensitive to penicillins [43, 44]. *L. paracasei* I-N-10 was also susceptible to tetracycline (MIC ≤ 4) and to gentamicin (MIC ≤ 32). Lactobacilli are generally susceptible to protein synthesis inhibitors as tetracycline and aminoglycosides as gentamicin [43, 45, 46]. Moreover, it was reported that phenotypic resistance to tetracycline is rarely found in *L. paracasei* species [47].

Resistance of *Lactobacillus* species to chloramphenicol as it was observed with *L. paracasei* I-N-10 strain was previously described [46, 48, 49].

Vancomycin resistance has been reported in *Lactobacilli* strains as an intrinsically resistance and chromosomally encoded [42, 43].

Proteolysis of β -casein and evaluation of potential application in milk allergy

Allergic reactions to foods are adverse reactions to individual food components, especially proteins. Cow's milk allergy affects in developing countries up to 8 % of children below 3 years age. Most studies revealed that caseins and β -lactoglobulin are main allergens in bovine milk [50]. Milk allergy is mediated by specific IgE antibodies directed toward specific milk proteins epitopes. Different technologies process, for example, enzymatic processing, have been developed to provide hydrolyzed formulae with low risk of allergy reactions. Numerous reports confirm that some LAB strains are able to decrease the number of epitopes by proteolytic cleavage during fermentation process and consequently reduce the antigenic response to milk proteins [17–19]. For these reasons, starters as *Lactobacillus* strains with beneficial properties able to reduce the allergenicity of fermented milk products are of great interest.

To evaluate the potential role of the strain *L. paracasei* I-N-10 in decrease in immuno-reactivity of β -casein, we performed LC MS/MS analysis of peptides released after proteolysis of this casein by cells of the strain in order to evaluate whether the proteolysis sites are situated on IgE-binding epitopes.

Figure 5 shows the sequence of bovine β -casein with the 4 major epitopes (L16-Q39, R25-H50, F52-I74, and T154-V173) previously reported [51, 52]. After mass spectrometry analysis, 7 peptides of sizes ranging from 8 to 13 amino acids were characterized. Three peptides (Q34-T41, D43-H50, and H145-M156) resulted from the splitting of peptide bonds located in 2 of the major cited epitopes (R25-H50 and T154-V173). The cleavages sites of *L. paracasei* I-N-10 protease(s) occur preferentially at proline and glutamic acid what remains in accordance with what was observed by Hébert et al. [53], when they characterized the pattern of α _{S1}- and β -caseins breakdown and release of bioactive peptides by cell-envelope proteinase from *L. delbrueckii* subsp. *lactis* CRL 581. The preference to cleave proline exclusively from the C-side is typical for prolyl endopeptidases and serine proteases widely distributed in bacteria, which can be used also to hydrolyze proline-rich prolamines [54].

The resistance of epitope F52-I74 to hydrolysis by proteases of *L. paracasei* I-N-10 may be due to its hydrophobic nature and to the presence of phosphoserine residues, as phosphorylated peptides were shown to be much more resistant to hydrolysis by peptidases [55].

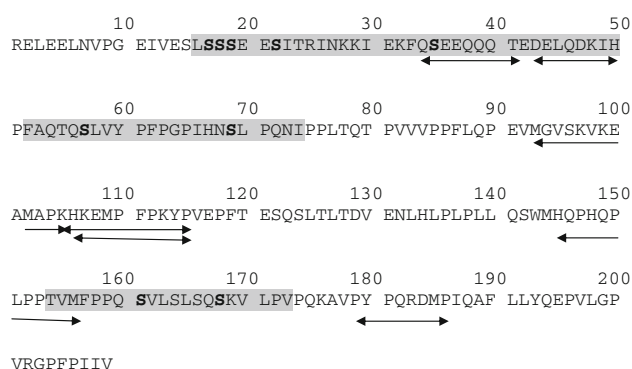


Fig. 5 β -Casein sequence and peptides released by the proteolytic action of *Lactobacillus paracasei* I-N-10 after 24-h incubation at 37 °C, as indicated by arrows. The peptides marked by a shadow refer to the main 4 epitopes reported by Cerecedo et al. [51] and Lin et al. [52]. The sequence uses the one-letter amino acid code, and the phosphorylated serines are indicated by S

Conclusions

In the present work, we investigated the proteolytic activities of LAB isolated from traditional Mongolian tarag. A collection of 33 isolates was screened for the production of proteolytic enzymes. One isolate has been identified as proteinase-producing strain, according to its ability to hydrolyze caseins. 16S rDNA fragment sequencing showed *L. paracasei* I-N-10 presented 99.9 % identity with the strain *L. paracasei* 1-4-2A. Hydrolysis of β -casein by proteases of *L. paracasei* I-N-10 affected some of the epitopes triggering the allergic reactions against β -casein. Further studies using an ELISA inhibition assay may clarify if any reduction in IgE binding can be observed, in order to conclude for the ability of *L. paracasei* I-N-10 to reduce allergenicity to milk proteins.

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