Proteolytic activity of *Enterococcus faecalis* VB63F for reduction of allergenicity of bovine milk proteins

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ABSTRACT

With the aim of screening proteolytic strains of lactic acid bacteria to evaluate their potential for the reduction of allergenicity of the major bovine milk proteins, we isolated a new proteolytic strain of *Enterococcus faecalis* (*Ent. faecalis* VB63F) from raw bovine milk. The proteases produced by this strain had strong activity against caseins (α_{S1} -, α_{S2} -, and β -casein), in both skim milk and sodium caseinate. However, only partial hydrolysis of whey proteins was observed. Proteolysis of Na-caseinate and whey proteins, observed after sodium dodecyl sulfate-PAGE, was confirmed by analysis of peptide profiles by reversed-phase HPLC. Inhibition of proteolysis with EDTA indicated that the proteases produced by *Ent. faecalis* VB63F belonged to the group of metalloproteases. The optimal conditions for their activity were 42°C and pH 6.5. The majority of assessed virulence genes were absent in *Ent. faecalis* VB63F. The obtained results suggest that *Ent. faecalis* VB63F could be efficient in reducing the immunoreactivity of bovine milk proteins.

Key words: protein hydrolysis, milk allergy, casein, whey protein

INTRODUCTION

Cow milk allergy (CMA) affects approximately 2.5% of children under 3 yr of age and represents 9% of all diagnosed cases of food allergies. It is estimated that about 80% of the allergic children overcome the problem in the adulthood. However, 20% of these patients remain allergic for life (Cocco et al., 2003; Gaudin et

al., 2008). Recent studies indicate that the frequency of CMA persistence to older ages is increasing (Pasalacqua et al., 2012; Feldman and Bird, 2014). The symptoms of CMA vary among patients and include gastrointestinal and skin problems, asthma, rhinitis, and, in extreme cases, anaphylaxis (Gaudin et al., 2008; Bu et al., 2013). Cow milk allergy is an immunological reaction related to the recognition and binding of IgE to specific epitopes in milk proteins (Cocco et al., 2003; Claeys et al., 2013). β -Lactoglobulin is the main milk protein recognized by IgE, accounting for 80% of allergy cases and identified as the main agent of CMA development (Pescuma et al., 2012; Claeys et al., 2013). However, milk caseins (α_{S1} -, α_{S2} -, β -CN) also play a relevant role in the development of CMA (Docena et al., 1996; Natale et al., 2004; Restani et al., 2009; Bu et al., 2010; El-Ghaish et al., 2010), and the linear epitopes present in this protein fraction of milk are related to the persistence of allergy and the occurrence of more severe symptoms (Vila et al., 2001).

Several strategies and technological approaches have been undertaken to reduce bovine milk allergenicity. Enzymatic and heating treatments have been used to induce modifications in the proteins' main epitopes and reduce their capability to bind to specific IgE (Taheri-Kafrani et al., 2009). Also, the combination of microwave irradiation and enzymatic proteolysis has yielded good results in reduction of immunoreactivity of β -LG (El Mecherfi et al., 2011). High temperatures can induce structural modifications in conformational epitopes, leading to loss of their binding capability. However, linear epitopes are unaffected by these modifications and remain able to bind to specific IgE. Because milk proteins have both conformational and linear epitopes, heating treatments exert a small effect on the immunoreactivity of milk, mainly in its casein fraction (Restani et al., 2009; Bu et al., 2013).

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Another alternative to reduce the immunoreactivity of milk proteins is the reduction of the molecular mass of the principal milk allergens and cleavage of the allergic epitopes by hydrolysis during microbial fermentation (Claeys et al., 2013). Milk is poor in free amino acids; therefore, microbial proteolytic enzymes play an important role in the generation of the EAA required for the growth of microorganisms in this food matrix (Lozo et al., 2011).

Lactic acid bacteria (**LAB**) are part of the microbiota of milk and possess a complex proteolytic metabolism, by means of proteinases, peptidases, and transport enzymes. Some strains of LAB produce proteases able to hydrolyze the epitopes of the proteins responsible for the development of CMA, reducing their allergenic potential (Bu et al., 2010; El-Ghaish et al., 2011; Pescuma et al., 2015). Several authors have reported that the treatment of caseins and whey protein fractions with proteolytic LAB can reduce the antigenic response of these proteins and suggest the inclusion of such hydrolysates in the manufacture of hypoallergenic formulations and dairy products (Bertrand-Harb et al., 2003; Cocco et al., 2003; Peñas et al., 2006; Pescuma et al., 2010, 2011).

Interest is increasing in the study of new LAB applicable in the development of novel probiotic and functional foods (Mozzi et al., 2013; Pescuma et al., 2015). Considering that functional foods are those that, in addition to their nutritional requirements, are beneficial for health (Koch et al., 2014), hypoallergenic fermented dairy products can be included in this group of foods (Pescuma et al., 2011). However, the reduction of immunoreactivity of milk proteins by the use of LAB depends on the strain and the process conditions (Kleber et al., 2006; Bu et al., 2010). This fact has stimulated more research on new proteolytic LAB strains able to hydrolyze the milk proteins responsible for CMA for application in the design of novel hypoallergenic dairy products (El-Ghaish et al., 2011). The aim of this study was to screen for novel proteolytic LAB strains in raw bovine milk, targeting caseins (α_{S1} -, α_{S2} -, and β -CN) and whey proteins, to identify the most promising strains, optimize the proteolytic activity, verify the absence of virulence factors, and evaluate the potential application in reduction of the allergenicity of bovine milk proteins.

MATERIALS AND METHODS

Isolation of Proteolytic LAB

Lactic acid bacteria were isolated from raw bovine milk samples obtained from a local farm in Nantes,

France. For isolation of proteolytic LAB, milk samples were submitted to serial 10-fold dilutions in 0.85% (wt/vol) NaCl solution and plated onto de Man, Rogosa and Sharpe (MRS) agar (Oxoid, Basingstoke, UK) and M17 agar (Oxoid), supplemented with 20% UHT skim milk (Délisse, Abbeville, France), as described by Ahmadova et al. (2011). Plates were incubated at 30°C, 37°C, or 42°C for 48 h, and examined for the presence of isolated colonies presenting clear surrounding halos, indicating potential production of proteolytic enzymes (Pailin et al., 2001). Colonies presenting these features were selected, transferred to M17 broth, streaked on M17 agar to obtain pure cultures, and then submitted to Gram staining and catalase test. Gram-positive and catalase-negative isolates were considered as LAB and stored at -80°C in M17 broth, containing 20% (wt/vol) glycerol (Sigma-Aldrich, Munich, Germany) as cryoprotector, for further characterization. Before use, the isolates were reactivated twice in UHT skim milk and then once in M17 broth.

Confirmation of Proteolytic Activity

Confirmation of proteolytic activity was performed by fermentation of UHT skim milk, according to El-Ghaish et al. (2010). Briefly, overnight cultures of each isolate in M17 broth were centrifuged at $10,000 \times g$ for 5 min at 4°C, and the pellets were resuspended in sterile Na-phosphate buffer (100 mM, pH 7.0). The suspensions were inoculated (5% vol/vol) in UHT skim milk and incubated at 37°C for 24 h (test samples). Resulting milk proteins and peptides were separated by SDS-PAGE, carried out according to Laemmli (1970). Controls consisted of UHT skim milk added to the same volume of sterile M17 broth. *Enterococcus faecalis* HH22 (El-Ghaish et al., 2010) was used as a positive control of proteolytic activity. The occurrence and extension of proteolysis was confirmed comparing the SDS-PAGE band profiles of test and control samples.

The SDS-PAGE was carried out in a vertical slab electrophoresis cell (Mini Protean 3 System, BioRad, Hercules, CA). The samples were diluted (1:1) in a solubilization buffer [50 mM Tris-HCl (Euromedex, Souffelweyersheim, France), pH 6.8, 4% SDS (Sigma-Aldrich), 20% glycerol, 3% 2-mercaptoethanol (Sigma-Aldrich), and 0.07% bromophenol blue (Sigma-Aldrich)] and heated at 60°C for 5 min. Heated samples were centrifuged ($10,000 \times g$ for 5 min at 25°C) and the supernatant was further diluted in the solubilization buffer (1:5, vol/vol) to achieve a convenient concentration of proteins. This mixture was heated at 100°C for 3 min, and loaded onto 12% polyacrylamide gels; the proteins were separated by electrophoresis in the following con-

ditions: 10 mA on the stacking gel (7.8% acrylamide) and 20 mA on the separation gel (12% acrylamide). Migration buffer was composed of 50 mM Tris, 0.384 M glycine (Euromedex), and 0.1% SDS. After electrophoresis, the gels were stained with Coomassie Brilliant Blue R250 overnight, destained for approximately 3 h with a solution composed of 30% (vol/vol) ethanol (Carlo Erba Reagents, Peypin, France) and 5% (vol/vol) acetic acid (Carlo Erba Reagents) and scanned with an Image Scanner III (GE Healthcare, Piscataway, NJ) for visualization of the bands.

Analysis of the Proteolytic LAB Strains

The 51 isolates, which had confirmed proteolytic activity against milk proteins, were submitted to random amplification of polymorphic DNA (RAPD)-PCR analysis for determination of similarity, using the primers E1 and E2 (Kirilov et al., 2011) and M13 (Rossetti and Giraffa, 2005), as described in Table 1. Total DNA of each isolate was extracted using the Bacterial DNA commercial kit (Omega Bio-Tek, Norcross, GA), and the concentration of DNA was measured by determining the ratio between the absorbance (A) at 260 and 280 nm (A_{260}/A_{280}), using a spectrophotometer (Bio-Photometer, Eppendorf, Westbury, NY).

Amplifications of DNA were carried out in reaction mixtures of 25 µL (final volume) containing 40 ng of DNA template, 0.2 µmol of primer, and 4.4 µL of 5× BioAmp master mix (BioFidal, Lyon, France). The amplification program for primers E1 and E2 consisted of a first denaturation step at 94°C for 5 min, 4 cycles of 94°C for 45 s (denaturation), 30°C for 2 min (annealing),

and 72°C for 30 s (extension), followed by 30 cycles of 94°C for 5 s (denaturation), 36°C for 30 s (annealing), and 72°C for 40 s (extension), with a final extension step at 72°C for 10 min. The amplification program for primer M13 consisted of a first denaturation step at 94°C for 2 min, followed by 40 cycles of 94°C for 30 s (denaturation), 42°C for 20 s (annealing), and 72°C for 2 min (extension), with a final extension step at 72°C for 10 min. All amplifications were performed in a Touchgene gradient thermocycler (Techno, Stone, UK).

The amplified products were separated by electrophoresis at 100 V on 1.5% (wt/vol) agarose gels with ethidium bromide (0.5 mg/mL; Sigma-Aldrich), in 0.5× Tris-acetate-EDTA buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0; Sigma-Aldrich) and observed under UV light. The strains presenting different profiles by RAPD-PCR analysis were submitted to identification through 16S rDNA amplification.

Identification of Proteolytic Strains

Total DNA of proteolytic LAB strains was used as template for amplification of the 16S rDNA gene by PCR with universal primers fD1 and rD1 (Table 1), according to Weisburg et al. (1991). The reaction mixture (50-µL final volume) was composed of 40 ng of DNA template, 0.5 µmol of each primer, and 8.6 µL of 5× BioAmp master mix (BioFidal). The amplification program included a first denaturation step at 95°C for 2 min, followed by 40 cycles of: 95°C for 30 s (denaturation), 45°C for 30 s (annealing), and 72°C for 30 s (extension) with a final extension step at 72°C for 7 min. Amplification was done in a Touchgene gradient

Table 1. Primers used in this study

Target	PCR primer (5'–3')	Reference
RAPD ¹	E1: TCACGCTGCA	Kirilov et al. (2011)
	E2: TCACGCTGCA	
	M13: GAGGGTGGCGTTCT	
16S rDNA	fD1: AGAGTTTGATCCTGGCTCAG	Rossetti and Giraffa (2005) Weisburg et al. (1991)
	rD1: TAAGGAGGTGATCCAGGC	
asa1	ASA 11: GCACGCTATTACGAACATATGA ASA 12: TAAGAAAGAACATCACCACGA	Vankerckhoven et al. (2004)
ace	ACE F: GAATTGAGCAAAAGTTCAATCG ACE R: GTCTGTCTTTTCACTTGTTTC	Ben Omar et al. (2004)
efaAfs	TE5: GACAGACCCTCACGAATA TE6: AGTTCATCATGCTGTAGTA	Eaton and Gasson (2001)
esp	ESP 14F: AGATTTTCATCTTTGATTCTTG4 ESP 12R: AATTGATTCTTTAGCATCTGG	Vankerckhoven et al. (2004)
cylA	CYT I: ACTCGGGGATTGATAGGC CYT IIb: GCTGCTAAAGCTGCGCTT	Vankerckhoven et al. (2004)
cylB	cylB1: AAGTACACTAGTAGAACTAAGGGA cylB2: ACAGTGAACGATATAACTCGCTATT	Semedo et al. (2003)
gelE	GEL 11: TATGACAATGCTTTTTTGGGAT GEL12: AGATGCACCCGAAATAATATA	Vankerckhoven et al. (2004)

¹Rapid amplification of polymorphic DNA.

thermocycler, and the sequencing of the amplified fragments was carried out by MilleGen sequencing service (Labège, France). The obtained sequences were compared with those available in GenBank database, using the Basic Local Alignment Search Tool (BLAST) at the National Center of Biotechnology Information website (<http://www.ncbi.nlm.nih.gov/>).

Investigation of Virulence Genes

The safety of the 9 identified proteolytic LAB strains for their application in food was assessed by investigation of the presence of virulence genes *asa1* (aggregation substance), *ace* (adhesin of collagen protein), *esp* (enterococcal surface protein), *efaAfs* (cell wall adhesin), *cylA/B* (cytolysins), and *gelE* (gelatinase). Tests were carried out by PCR using the specific primers described in Table 1. The reaction mixtures (25- μ L final volume) contained 40 ng of DNA template, 0.2 μ mol of each primer, and 4.4 μ L of 5 $\times$ BioAmp master mix. The amplifications were carried out in a Touchgene gradient thermocycler as follows: 94°C for 15 min (first denaturation step), 35 cycles of 94°C for 1 min (denaturation), annealing step for 1 min at 58°C (*cylA*), 52°C (*cylB*), 54°C (*efaAfs*), 45°C (*gelE*), and 56°C (*asa1*, *ace*, and *esp*), and extension at 72°C for 1 min, with a final extension step at 72°C for 5 min. The amplified fragments were analyzed by electrophoresis at 100 V in 1% (wt/vol) agarose gels with ethidium bromide (0.5 mg/mL), in 0.5 $\times$ Tris-acetate-EDTA buffer pH 8.0 and observation under UV light. Genomic DNA from *Enterococcus faecalis* MMH594, harboring the target virulence genes, was used as positive control (Shankar et al., 2001).

Proteolytic Activity in a Nonproliferative Cell System

The proteolytic LAB strains were tested for the capability to hydrolyze Na-caseinate and whey proteins in the nonproliferative cell system described by El-Ghaish et al. (2010). Overnight cultures of each proteolytic LAB strains were obtained in M17 broth. The cells were removed from the medium by centrifugation (10,000 $\times$ g, 5 min, 4°C) and resuspended in the same volume of sterile saline solution (0.85% NaCl, wt/vol). Proteinase production was induced by surface-plating the suspensions (200 μ L) on milk-citrate agar, composed of UHT skim milk (44%, vol/vol), Na-citrate (0.8%, wt/vol; Sigma-Aldrich), yeast extract (0.1% wt/vol; Fluka, Sigma-Aldrich), glucose (0.5%, wt/vol; Sigma-Aldrich), and agar (1.5%, wt/vol; Fluka, Sigma-Aldrich; Fira et al., 2001). After incubation at 37°C for 24 h, sterile saline solution (0.8% NaCl), supplemented with 10 mM CaCl₂, was added to the plates and the cells were col-

lected with the help of a Drigalski loop. The cell suspensions were washed twice in the same diluent (10,000 $\times$ g, 5 min, 4°C) and resuspended in Na-phosphate buffer (100 mM, pH 7.0) to a final optical density at 600 nm of 20.

Proteolytic activity was tested by mixing the cell suspensions (1:1, vol/vol) with purified fractions of milk proteins (Na-caseinate or denatured whey proteins, all of analytical grade and obtained at a laboratory scale after precipitation at pH 4.6). Both Na-caseinate and whey proteins were solubilized in Na-phosphate buffer (100 mM, pH 7.0) and brought to a concentration of 12 mg/mL. The solution of whey proteins was subjected to a denaturation step by heating at 80°C for 30 min, before mixing with LAB cells. The mixtures of LAB cells and protein substrates were incubated at 37°C for 24 h and centrifuged (10,000 $\times$ g, 5 min, 4°C), and the supernatants were analyzed by SDS-PAGE. The LAB strain able to cause the highest reduction in the intensity of the color of the bands, representing proteins from Na-caseinate and whey fractions, was considered to display the best proteolytic activity and selected for further experiments.

The protein hydrolysis assays performed in nonproliferative cell system included negative controls, prepared by mixing the protein substrates with Na-phosphate buffer in a 1:1 (vol/vol) ratio and incubating in the same conditions as test samples.

Effect of Temperature and pH on Proteolytic Activity

The tests were performed in the nonproliferative cell system, using either Na-caseinate or denatured whey proteins as substrates. The influence of temperature on proteolytic activity was evaluated by mixing the cell suspension of the selected LAB strain with the substrates in a 1:1 (vol/vol) ratio and incubating at 30°C, 37°C, or 42°C for 24 h. To determine the effect of pH, the cell suspension was prepared in Na-phosphate buffer (100 mM) with different pH values (6.0, 6.5, and 7.0) and mixed with the substrates (1:1, vol/vol), solubilized in Na-phosphate buffer (100 mM) at the corresponding pH. The mixtures were incubated at 37°C for 24 h. Protein hydrolysis and peptide formation were observed submitting the mixtures to SDS-PAGE, as described in the section Confirmation of Proteolytic Activity.

Effect of Inhibitors on Proteolytic Activity

The type of proteinase produced by the selected LAB strains was determined by testing the effect of protease inhibitors on proteolytic activity in the nonproliferative

cell system. The tests were carried out under the optimal conditions of pH and temperature for the proteolytic activities determined as described in the subsection Effect of Temperature and pH on Proteolytic Activity (42°C, pH 6.5) and using Na-caseinate or denatured whey proteins as substrates. The cell suspensions were prepared in Na-phosphate buffer (100 mM, pH 6.5) and added of different protease inhibitors: EDTA (inhibitor of metalloproteases); iodoacetic acid (inhibitor of cysteine proteases); or 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Pefabloc; inhibitor of serine proteases); all reagents were from Sigma-Aldrich. All inhibitors were added to the cell suspensions to a final concentration of 10 mM. After incubation for 2 h at 37°C, the cell suspensions were mixed with the substrate solutions (Na-caseinate or denatured whey proteins) in a 1:1 (vol/vol) ratio, incubated at 42°C for 24 h, and analyzed by SDS-PAGE.

Kinetics of Proteolysis

The kinetics of hydrolysis of Na-caseinate or denatured whey proteins by the selected LAB strain was studied in the nonproliferative cell system, at pH 6.5. The cell suspensions were mixed with protein substrates (1:1, vol/vol) and incubated at 42°C. Samples were collected after 3, 6, 9, or 24 h of incubation and analyzed by SDS-PAGE for the observation of proteins hydrolysis and peptide formation.

Proteins Hydrolysis and Peptide Profiles Assessment in RP-HPLC

The extension of protein hydrolysis and the profile of peptides generated by the treatment of Na-caseinate or denatured whey proteins with the cell suspension of selected proteolytic LAB strain were analyzed by RP-HPLC. The proteolysis was carried out in the nonproliferative cell system at pH 6.5. The cell suspension was mixed with the substrate solutions (1:1, vol/vol) and incubated at 42°C for 24 h. Before injection in the column, the sample was mixed (1:1, vol/vol) with 9 M urea (Acros Organics, Geel, Belgium) and centrifuged ($12,000 \times g$ for 5 min at 4°C) to remove the cells. The supernatant was injected in a C₁₈ symmetry column (2.1 × 150 mm, Waters, Milford, MA) and the analysis was carried out in a HPLC System Alliance (Waters), under the following conditions: flow rate, 0.2 mL/min; solvent A, water 94.95%, acetonitrile 5.0%, trifluoroacetic acid (TFA) 0.05% (vol/vol/vol); solvent B, isopropanol 19.97%, acetonitrile 80%, TFA 0.03% (vol/vol/vol); all reagents were from Carlo Erba Reagents. Elution was performed with a linear gradient of solvent

B, ranging from 0 to 100%. Detection was performed at 220 nm, using a photodiode array detector (model 996, Waters), and the data were analyzed by using Empower software (Waters). The obtained protein and peptide profiles were compared with those obtained for control protein fractions.

RESULTS AND DISCUSSION

A collection of 200 isolates showed potential production of proteolytic enzymes. Sixty-five isolates were confirmed as LAB and selected for confirmation of proteolytic activity in skim milk. Of these, 51 isolates were able to hydrolyze at least one milk protein fraction, indicated by reduced color intensity of the bands in SDS-PAGE and by appearance of new bands with lower molecular weight compared with the control (data not shown).

The RAPD profiles of the 51 proteolytic isolates indicated that they belonged to 9 strains. Using primers M13 and E2, 3 and 6 different profiles were obtained, respectively, whereas amplification with primer E1 generated identical fragments for all isolates. One isolate of each strain was submitted to amplification and sequencing of 16S rDNA, which resulted in 99% identity with the 16S rRNA sequences reported for *Enterococcus faecalis* strains, such as *Ent. faecalis* S4-15 (accession number: KC478511.1), *Ent. faecalis* KDLLL4-1 (accession number: KC454284.1), and *Ent. faecalis* CNM506 12 (accession number: KC699226.1), indicating that all 9 isolates were *Ent. faecalis*. These results agree with literature data in which *Enterococcus* is the most common LAB genus in raw milk microbiota, with *Ent. faecalis* and *Ent. faecium* being the most common enterococcal species in milk and dairy products (Quigley et al., 2013). *Enterococcus* is known to possess a very efficient proteolytic system, contributing to the development of sensorial characteristics in cheeses and other fermented dairy products (Franz et al., 1999; Foulquié Moreno, et al., 2006; Serio et al., 2010; Ahmadova et al., 2011).

The PCR assays indicated that the 9 isolates possessed the genes *asa1* (aggregation substance), *ace* (adhesin of collagen protein), and *gelE* (gelatinase), and all were negative for the genes *esp* (enterococcal surface protein), *efaAfs* (cell wall adhesin), *cylA* (cytolysin A), and *cylB* (cytolysin B). Checking for these virulence genes is of utmost importance as enterococci can be the causative agents of infections in hospitalized patients and nosocomial bloodstream infections (Vankerhoven et al., 2008). Moreover, the investigation of virulence factors in LAB for food applications is mandatory as they may contain several determinants of pathogenic-

ity received from other bacterial species via horizontal gene transfer. Several virulence factors were already described in *Enterococcus*. Cytolysin remains one of the most important and studied virulence traits attributed to enterococci, and the molecular screening of *cyl* genes is recommended for hemolytic and nonhemolytic strains (Morandi et al., 2013). It is important to note that none of the isolates studied here presented the genes encoding cytolysins A and B. However, 2 genes encoding adhesion-associated proteins were found: *asa1* and *ace*. Other authors have reported a high incidence of *asa1* and *ace* in *Ent. faecalis* strains isolated from dairy products, although none of these genes has been clearly and unequivocally associated with clinical isolates (Martín-Platero et al., 2009; Ahmadova et al., 2011). Another virulence gene found in all strains was *gelE*, which encodes a zinc metalloprotease that has been shown to contribute to *Ent. faecalis* virulence in some animal models. However, the presence of the *gelE* gene does not imply that the strain will express gelatinase activity (Eaton and Gasson, 2001; Qin et al., 2001; Semedo et al., 2003). The *gelE* gene is frequently found in proteolytic strains isolated from dairy and meat products (Lopes et al., 2006; Ahmadova et al., 2011).

Tests performed with Na-caseinate or denatured whey proteins indicated that the 9 strains shared similar proteolytic activity, with the ability to hydrolyze both protein substrates, albeit to different extents. The highest proteolytic activity was observed against Na-caseinate fractions (β -, α_{S1} -, and α_{S2} -CN), whereas only weak activity was observed against the denatured whey proteins (α -LA and β -LG). Qualitative comparison of staining intensity of the bands indicated that *Ent. faecalis* VB63F was the most active strain.

Figure 1. Sodium dodecyl sulfate-PAGE showing the Na-caseinate hydrolysis profiles caused by proteolytic strains (37°C, 24 h, pH 7.0). C- = negative control (Na-caseinate only); lanes 7 to 71 = test samples (Na-caseinate and the proteolytic strain).

All isolates displayed weak activity against α -LA and β -LG (data not shown). However, when tested for proteolysis of Na-caseinate, *Ent. faecalis* VB63F was the most active against β -, α_{S1} -, and α_{S2} -CN (Figure 1). This strain was considered the most active and was selected for characterization of proteolytic activity.

Hydrolysis of Na-caseinate and denatured whey proteins fractions was influenced by pH and temperature. Proteases produced by *Ent. faecalis* VB63F were more active at 42°C than at 30°C or 37°C, for both protein substrates (Figures 2A and 2B). When hydrolysis occurred at 30°C, only the band corresponding to β -CN showed reduced staining intensity, whereas the bands representing α_{S1} - and α_{S2} -CN remained unchanged and were equal to those of the negative control. The staining intensity of all bands representing Na-caseinate was reduced at 37°C, suggesting that β -, α_{S1} -, and α_{S2} -CN were at least partially hydrolyzed at this temperature. However, at 42°C, the reduction in the staining intensity of the bands of Na-caseinate was stronger than that observed at 37°C. Moreover, β - and α_{S2} -CN were hydrolyzed completely, as no electrophoretic band could be detected. In all tested temperatures, new bands of low molecular weight appeared, indicating the release of small peptides. In the case of denatured whey proteins, no hydrolysis was observed at 30°C or 37°C, and a slight reduction in the staining intensity of the bands was observed when the testes were carried out at 42°C, indicating partial hydrolysis of these proteins. These results agree with what was found by El-Ghaish et al. (2010), who observed that the extent of hydrolysis of bovine milk proteins by different strains of *Ent. faecalis* was higher at 42°C than at other temperatures.

The pH did not influence the proteolytic activity of the proteases produced by *Ent. faecalis* VB63F (Figure 3A), as β -, α_{S1} -, and α_{S2} -CN were hydrolyzed at all tested pH. However, the best results were observed at pH 6.5. All Na-caseinate fractions were intensively hydrolyzed at pH 6.0 and 7.0; however, α_{S1} -casein was somewhat resistant. The casein electrophoretic bands disappeared at pH 6.5, indicating total hydrolysis of β -, α_{S1} -, and α_{S2} -CN. In case of denatured whey proteins, the best results were obtained when proteolytic activity was tested at pH 6.5 and pH 7.0 (Figure 3B), when a reduction in the staining intensity of the bands representing α -LA and β -LG was observed, indicating partial hydrolysis of these proteins. At pH 6.0, no hydrolysis was observed. Because pH 6.5 yielded the best results for both Na-caseinate and denatured whey proteins, this pH was selected as the best one for the studies of the activity of the proteases produced by *Ent. faecalis* VB63F. Other authors also reported that proteolysis

Figure 2. Sodium dodecyl sulfate-PAGE showing the hydrolysis profiles of Na-caseinate (A) and denatured whey proteins (B), caused by *Enterococcus faecalis* VB63F at 30°C, 37°C, and 42°C (24 h, pH 7.0). Lane C- = negative controls [Na-caseinate (A) or denatured whey proteins (B)]; lane 63 = samples treated with *Ent. faecalis* VB63F.

of *Ent. faecalis* was more effective at pH 6.5 or close to neutral (El-Ghaish et al., 2010; Ahmadova et al., 2011).

The metalloprotease inhibitor EDTA strongly suppressed the hydrolysis of both Na-caseinate and denatured whey proteins by *Ent. faecalis* VB63F (Figure 4A and B, respectively). Neither iodoacetic acid nor Pefabloc affected proteolytic activity, indicating that neither cysteine nor serine proteases were involved. Others studies have reported that strains of *Ent. faecalis* produce metalloproteases and, if other types of proteases were present, they played a minor role in the hydrolysis of milk protein fractions (El-Ghaish et al., 2010; Ahmadova et al., 2011).

Concerning the kinetics of proteolysis of β -, α_{S1} -, and α_{S2} -CN, β -LG, and α -LA by *Ent. faecalis* VB63F,

carried out under optimal conditions of pH and temperature, the extent of hydrolysis varied according to substrate (Figure 5A). β -Casein was the main protein targeted by the proteases of *Ent. faecalis* VB63F—the band corresponding to the native form disappeared completely from the gel in the first hours at 42°C. The second target was α_{S2} -CN, which was completely hydrolyzed after 9 h. At the end of the incubation period, the band representing the native form of α_{S1} -CN was also greatly reduced. These results indicate that the proteolytic enzymes produced by *Ent. faecalis* VB63F have a strong activity against Na-caseinate fractions of milk proteins. In case of denatured whey proteins (Figure 5B), hydrolysis occurred only after 24 h and the reduction in the staining intensity of the bands

Figure 3. Sodium dodecyl sulfate-PAGE showing the hydrolysis profiles of Na-caseinate (A) and denatured whey proteins (B), caused by *Enterococcus faecalis* VB63F at pH 6.0, 6.5, and 7.0 (37°C, 24 h). Lane C- = negative controls [Na-caseinate (A) or denatured whey proteins (B)]; lane 63 = samples treated with *Ent. faecalis* VB63F.

Figure 4. Sodium dodecyl sulfate-PAGE showing the hydrolysis profiles of Na-caseinate (A) and denatured whey proteins (B), caused by *Enterococcus faecalis* VB63F (42°C, 24 h, pH 7.0) in the presence of protease inhibitors. C- = negative controls [Na-caseinate (A) and denatured whey proteins (B)]; C+ = positive control [Na-caseinate (A) and denatured whey proteins (B) inoculated with proteolytic strain]; lane 63 = samples treated with *Ent. faecalis* VB63F; EDTA = metalloprotease inhibitor; Iodo = iodoacetic acid (cysteine protease inhibitor); Pefa = Pefabloc (serine protease inhibitor). All inhibitors were from Sigma-Aldrich (Munich, Germany).

corresponding to α -LA and β -LG were weaker than those of Na-caseinate. These results suggest that the proteolytic enzymes produced by *Ent. faecalis* VB63F have poor activity against whey proteins and are only able to partially hydrolyze α -LA and β -LG.

The HPLC analysis of Na-caseinate and whey protein fraction proteolysis by *Ent. faecalis* VB63F, after 24 h and under optimal conditions of pH and temperature, confirmed the results observed by SDS-PAGE (Figure 6A). The peaks representing the native forms of the caseins disappeared and smaller peaks appeared, suggesting the release of medium-sized, less-hydrophobic peptides. The HPLC profiles of denatured whey proteins showed that α -LA and β -LG were still present in treated samples (Figure 6B), but the appearance of less-hydrophobic peaks suggested that whey proteins were partially hydrolyzed.

Our results indicate that *Ent. faecalis* VB63F has strong proteolytic activity against bovine caseins. Although β -LG is recognized by many authors as the main allergen in bovine milk (Pescuma et al., 2012), caseins also play an important role in CMA development, because many allergic patients present casein-specific IgE (Docena et al., 1996; Bu et al., 2013). In addition, it has been demonstrated that the persistence of milk allergy is associated with the presence of specific IgE that recognize linear epitopes (present in casein fractions), rather than the conformational epitopes that are present in β -LG (Vila et al., 2001; Järvinen et al., 2002). Järvinen et al. (2002), studying persistence of cow milk allergy, noted that patients with symptoms of persistent milk allergy recognize a greater number of sequential (linear) milk-protein epitopes on α _{S1}- and β -CN, compared with patients who develop clinical

Figure 5. Sodium dodecyl sulfate-PAGE showing the hydrolysis profiles of Na-caseinate (A) and denatured whey proteins (B), caused by *Enterococcus faecalis* VB63F after 3, 6, 9, and 24 h at 42°C (pH 6.5). C- = negative controls [Na-caseinate (A) and denatured whey proteins (B)]; M = molecular weight marker; BLG = β -LG, ALA = α -LA.

Figure 6. Reverse phase-HPLC profiles of milk proteins resulting from hydrolysis of Na-caseinate (A) and denatured whey proteins (B), caused by *Enterococcus faecalis* VB63F. C = negative control (not treated with *Enterococcus faecalis* VB63F); VB63F = test samples (treated with *Enterococcus faecalis* VB63F).

tolerance to milk. Similar results were reported by Sicherer and Sampson (1999). Those authors found that patients with persistent allergy (over 9 yr of age) had significantly elevated levels of casein-specific IgE. Thus, the study of new proteolytic LAB strains able to hydrolyze the casein fractions of milk could help reduce the allergenicity of bovine milk proteins and provide interesting data, which could lead to the development of new hypoallergenic dairy products.

CONCLUSIONS

Our data indicate that *Ent. faecalis* VB63F has good proteolytic activity against caseins. This interesting trait, combined with absence of virulence factors, raise the possibility of applying this strain to reduce the allergenicity of bovine milk proteins. Further examination of the specific IgE capability to bind the Na-caseinate hydrolysates will indicate the level of reduction in the immunoreactivity of these milk protein fractions.

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