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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 (Passalacqua 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 × *g* for 5 min at 4°C, and the pellets were resuspended in sterile Na-phosphate buffer (100 m*M*, 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 m*M* 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 × *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-