

Allergenicity reduction of bovine milk β -lactoglobulin by proteolytic activity of *Lactococcus lactis* BMC12C and BMC19H isolated from Iranian dairy products

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ABSTRACT

Nowadays health benefits of bioactive food constituents, known as probiotic microorganisms, are a growing awareness. Cow's milk is a nutritious food containing probiotic bacteria. However, milk allergenicity is one of the most common food allergies. The milk protein, β -lactoglobulin (BLG), is in about 80% of all main cases of milk allergies for children and infants. 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 new proteolytic strains of *cocci lactis acid* bacteria from traditional Iranian dairy products. The proteases produced by these strains had strong proteolytic activity against BLG. Proteolysis of BLG, observed after sodium dodecyl sulfate-PAGE, was confirmed by the analysis of the peptide profiles by reversed-phase HPLC. The two isolates were submitted to 16S rDNA sequencing and identified as *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *hordniae*. The competitive ELISA experiments confirmed that these isolates, with high proteolytic activity, reduce significantly the allergenicity of BLG. Accordingly, these isolates can reduce the immunoreactivity of bovine milk proteins, which can be helpful for the production of low-allergic dairy products.

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1. Introduction

The food allergy has been known as the sixth human health problem by the World Health Organization (WHO) [1]. Many academies in the world have decided to share and expand information on the food allergic diseases due to their increasing role in human life. Although any food can stimulate immune response reaction, nearly few foods are responsible for the majority of food-induced allergic reactions. These foods include milk, peanut, fish, crustacean shellfish, tree nuts, wheat, soy and egg [2]. One of the most abundant food allergens is cow's milk [3,4].

Cow's milk allergy (CMA), which is very common in infants and young children, is an immunological reaction related to the recognition and binding of IgE to specific epitopes in milk proteins [5–7]. The prevalence of allergy to milk proteins varies between 3.0 and 7.5% among populations of different countries. It is estimated that about 80% of the allergic children overcome the problem in the adulthood. However, the other 20% will remain allergic for life [8–12]. There are different clinical symptoms of CMA including digestive, skin, respiratory problems, and in extreme cases anaphylaxis [13–17].

Cow's milk contains 30–35 g protein per liter. The acidification of the milk to pH 4.6 results in the separation of proteins into 2 fractions: coagulum (curd), which contains approximately 80% of the cow's milk proteins (CMPs), and lactoserum (whey), which contains approximately 20% of the CMPs. The most important allergen proteins in cow's milk are β -Lactoglobulin (BLG) and α_{s1} -casein [18–22]. More than 50% of the individuals with CMA are sensitized to these proteins [23–26].

β -Lactoglobulin belongs to the lipocalin structural family. It is a small globular protein with a molar mass of 18.3 kDa [27–30]. BLG is known as a natural molecular nanocarrier. It has three possible binding sites for hydrophobic ligands, such as fatty acids and some vitamins [31–34]. The biological function of this protein has not yet been well determined. Studies of epitope mapping of BLG proposed several immunoreactive epitopes; residues 41–60, 102–124, 149–162 and 84–91 have been recognized as the common immunoreactive epitopes of the protein. Several other peptides are distributed all along the sequence of the protein [35]. BLG remains stable or break down into high molecular weight (MW) peptides in the acidic and proteolytic environment of the stomach, which maintain the IgE binding and T-cell stimulating properties [36,37]. However, many studies have been carried out to reduce the allergenicity of cow's milk proteins. Also, the better use of cow's milk in infant nutrition has been developed with the help of different technological processes [38–40]. The molecular basis of changes

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in allergenicity is the formation of new epitopes, better availability of hidden epitopes and inactivation or demolition of epitopes by denaturation of the natural allergen epitopes [41,42].

Various processing methods, such as enzymatic and heating treatments have been used to induce some modifications in the proteins' main epitopes and reduce their capability for binding to specific IgE, and therefore, reduce allergenicity of bovine milk proteins [43–45]. Furthermore, acidic pH values, high pressure, mutation, glycosylation and lactic acid fermentation have yielded good results in the reduction of immunoreactivity of BLG [46–50]. Fermentation is a common processing technology in the food industry. Fermented foods are capable of releasing bioactive peptides from principal food proteins by microbial enzymatic hydrolysis activity, and therefore, have a positive effect on the human health or physiology [51–55].

During microbial fermentation, lactic acid bacteria (LAB) produce proteolytic enzymes causing the degradation of milk proteins [56,57]. The milk digestibility can be influenced by the proteolysis of milk protein allergens by LAB fermentation and subsequently, the milk allergenicity decreases due to the hydrolysis of antigenic epitopes [58,59]. There are many evidences show that some LAB strains isolated from various dairy products from different parts of the world can reduce the allergenicity of bovine milk proteins especially β -Lactoglobulin [60–66]. A significant number of experiments on the potential of LABs in decreasing the antigenic effects of BLG confirm that the decrease in protein antigenicity varies with the LAB species and the conditions of fermentation process [65–67]. This fact has stimulated more researches with new proteolytic LAB strains able to hydrolyze the milk allergen proteins for application in the design of novel hypoallergenic dairy products. Therefore, in the present work, we isolated 112 strains from 27 samples of traditional Iranian dairy products. The aim of this study was to screen for novel proteolytic *cocci lactic acid* bacteria in raw bovine milk, targeting BLG, to identify the most promising strains, optimize the proteolytic activity, and evaluate the potential application in reduction of the allergenicity of bovine milk β -Lactoglobulin.

2. Materials and methods

2.1. Materials

Culture media were obtained from Difco Laboratories (USA). The chemicals were of analytical grade with the highest degree of purity obtained from Merck and Sigma–Aldrich chemical companies and were used without further purification. All solutions, prepared with double distilled water, were maintained at 4 °C until usage. BLG variant A (as a reference in all experiments) was obtained from homozygote cow's milk following the method of Maillart and Ribadeau Dumas [66]. The purity of BLG (>98%) was confirmed by high performance liquid chromatography (HPLC) and polyacrylamide gel electrophoresis (PAGE). A pool containing 21 sera from CMA patients with various symptoms were used for competitive enzyme-linked immunosorbent assay (ELISA) experiments. Total milk protein- and BLG-specific IgE concentrations were determined with the Phadia ImmunoCAP System. All sera had total milk protein-specific IgE concentrations between 18.6 and 49.1 kAUL⁻¹. The used sera were collected in the laboratory of immunology at the academic hospital (Tehran, Iran), and their use was approved by the internal ethical committee of the hospital. The sera from nonallergic patients were used as the negative control.

2.2. Isolation of *cocci lactic acid* bacteria from Iranian dairy products

Lactic acid bacteria used in this study were isolated from 27 samples of traditional Iranian dairy products from different regions of Iran. Milk samples were submitted to serial 10-fold dilutions in 0.9% (w/v) NaCl solution (Ringer solution) and mixed thoroughly. Serial dilutions (10^{-1} to 10^{-8}) were prepared, and 1 mL of the appropriate dilution was

pourplated onto M17 and de Man-Rogosa-Sharpe (MRS)-agar medium. Plates were incubated at 37 °C under microaerobic and anaerobic condition for 48 h. Colonies with the same appearance were selected and subcultured to MRS-agar to obtain pure cultures. Phenotypic identification was done by Gram staining, catalase tests and microscope examination of the cell morphology. The purified isolates were reconstituted in sterile skim milk (12.5%, w/v), supplemented with 30% (w/v) glycerol, and stored at –80 °C.

2.3. Screening the proteolytic activity of LABs

Preliminary detection of proteolytic enzymes production was performed by milk-agar technique. Different colonies were separately inoculated into the MRS-broth. Then, 100 μ L of the activated preculture was applied on the top of milk-citrate-agar (MCA) plates containing 4.4% skim milk powder (Délisse, UHT skim milk, France), 0.8% sodium citrate, 0.1% yeast extract, 0.5% glucose and 1.5% agar. The plates were incubated for 48 h at 37 °C prior to cell collection. Collected fresh cells were resuspended in 100 mM sodium phosphate buffer (pH 7.0) containing 5 mM CaCl₂ and brought to final optical density (OD₆₀₀) of 25 just before application. One volume of the last suspension was combined with an equivalent volume of the protein solution (5 mg/mL) taken as substrates for the proteolytic activity. Then, the mixture was incubated for 12 and 24 h at 37 °C in a shaker incubator. Controls were prepared by incubating equivalent protein solutions for the same periods without adding cells. At the end of incubation periods, cells were pelleted by centrifugation (10 min at 8000g) and the clear supernatant was analyzed by SDS–PAGE (12% polyacrylamide) and reversed phase high performance liquid chromatography (RP–HPLC). Finally, strains with the best proteolytic activity against β -lactoglobulin were selected to investigate their effect on the IgE binding and immunoreactivity properties of BLG, using competitive ELISA test.

2.4. SDS–PAGE analysis

Protein hydrolysates were analyzed on SDS–PAGE by loading 12% polyacrylamide gel on vertical slab electrophoresis cells. Briefly, protein hydrolysates were mixed in loading buffer (60 mM Tris–HCl pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.025% (w/v) bromophenol blue) and packed on 12% polyacrylamide gel containing 0.1% (w/v) SDS. Migration was done for about 1 h at 10 mA on the stacking gel and 20 mA on the running gel (for 1 gel), in a buffer containing 0.3% (w/v) Tris and 1.44% (w/v) glycine. The gels were stained with an aqueous solution containing 50% (v/v) ethanol, 10% (v/v) glacial acetic acid and 0.25% (w/v) Coomassie brilliant blue and destained with a solution containing 10% (v/v) ethanol and 7% (v/v) glacial acetic acid. The gels were scanned with Image scanner III (GE Healthcare, USA) and the intensity of the bands was quantified by image software. Analysis was performed using Fuji Film Image Gauge V3.0 software (Fuji Photo Film Co. Ltd., Japan).

2.5. RP–HPLC analysis

Protein hydrolysis and peptides profiles arising from enzymatic activities of the studied strains were analyzed by RP–HPLC. Supernatants, obtained after incubation of cells with different substrates in nonproliferative cells system, were mixed (1/1, v/v) with 6 M urea and centrifuged (12,000 g, 5 min) before injection to the column. 50 μ L of samples were separated on C₁₈ column (C₁₈, 4.6 $\times$ 150 mm, particle size of 5 μ m, Alltech Prevail, Grace, Illinois, USA) with a linear gradient (0.3 mL/min at 20 °C; solvent A: 10% v/v acetonitrile, 0.1% v/v trifluoroacetic acid; solvent B 90% v/v acetonitrile, 0.1% v/v trifluoroacetic acid). HPLC System: 1200 series Agilent HPLC, Agilent automatic injector (Agilent Technologies, Palo Alto,

CA), diode array detector set at 220 nm; EZ Chrom Elite software (version 3.3.1).

2.6. Competitive enzyme-linked immunosorbent assay (ELISA) experiments

Effect of proteolysis of BLG by *cocci lactic acid* bacteria on the immunoreactivity of the protein (IgE binding) was investigated using competitive ELISA experiment. The 96-well micro titration plates were pre-coated overnight with native BLG diluted to 5 µg/mL in PBS buffer. The plates were washed three times with PBS containing 0.1% (v/v) Tween-20 (PBS/T) and then saturated for 2 h with 250 µL of PBS/T containing 1% (w/v) polyvinyl alcohol (PBS/T/PVA). In parallel, IgE containing sera were incubated with increasing concentrations of native or hydrolysates BLG (0 to 250 µg/mL in 50 mM phosphate buffer pH 7.0). Each incubation mixture of 100 µL contained 5 µL of IgE serum. The IgE/BLG complexes were then added to the 96-well plates, pre-coated with native BLG, and incubated overnight at 4 °C. Unbound IgE (BLG complexes) were removed by washing the plates with PBS/T. IgE bound to the wells was identified by the addition of 100 µL of alkaline-phosphatase conjugated polyclonal anti-human IgE (Sigma) diluted in PBS/T/PVA (1:1000). After 2 h at 4 °C the plates were washed three times. Secondary antibody binding was quantified with the chromogenic substrate 4-methylumbelliferyl phosphate (4-MUP) diluted in 1 M Tris-HCl (1:5) with pH 9.8. After 90 min incubation at room temperature product fluorescence emission was measured on an FLx800 plate reader (BioTek Instruments) fitted with a 360 nm excitation filter and a 440 nm emission filter. The concentration of native or hydrolysate BLG (inhibitor) required to inhibit 50% of the total binding of serum IgE (IC₅₀) was calculated from the standard (inhibition) curves by relating fluorescence intensity to inhibitor concentrations. Fluorescence data were corrected by subtracting the fluorescence of the control with no antigen [67].

2.7. 16S rDNA amplification, identification and sequencing

Two gram-positive catalase-negative isolates, which have been confirmed to have the best proteolytic activity against BLG, with the greatest allergenicity reduction, were grown on MRS agar at 30 °C overnight. Then, genomic DNA from the aforementioned bacterial strains were extracted from the cultures with a TaKaRa minibest bacterial genomic DNA extraction kit (TaKaRa, Dalian, China), according to the manufacturer's instructions. The faecal samples were thawed on ice, and about 200 mg stool sample was weighed for each extraction. An E.Z.N.A.TM stool DNA isolation kit (Omega Bio-Tek, Doraville, CA) was used to extract bacterial DNA, according to the manufacturer's instructions and used as a template for 16S rDNA gene amplification. The concentration of DNA was measured by determining the ratio of the absorbance (A) at 260 and 280 nm (A₂₆₀/A₂₈₀), using a UV-Vis spectrophotometer, Carry-500 double beam (USA). Total DNA of proteolytic LAB strains was used as template for amplification of the 16S rDNA gene by PCR with universal primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-CGG TTA CCT TGT TAC GAC TT-3'). PCR amplifications were performed with a DNA thermal cycler [68]. The reaction mixture contains 5 µL of PCR buffer, 2 µL of MgCl₂, 1 µL of dNTPs, 1 U of Taq DNA polymerase, 1 µL of each primer, and 40 ng of DNA in a final volume of 50 µL. The PCR amplification program included a first denaturation step at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min (denaturation), 47 °C for 45 s (annealing) and 72 °C for 1 min (extension) with a final extension step at 72 °C for 10 min. Amplicons were analyzed on 1% (w/v) agarose gel with safe red view (1 µL/mL) in 0.5 × TAE buffer pH 8.2–8.4, for 30 min at 100 V and made visible by UV trans-illumination. DNA sequencing for 15 ng unknown samples was carried out by SeqLab sequencing service. The blast algorithm (<http://blast.ncbi.nlm.nih.gov>) was used to ascertain the most related sequence relatives in the NCBI nucleotide sequence database.

3. Results and discussion

3.1. Isolation of cocci lactic acid bacteria from Iranian dairy products

After several steps of purification by streaking on MRS agar, a collection of 112 isolates was screened from 27 samples of traditional Iranian dairy products. This collection showed a potential production of proteolytic enzymes, among which 48 isolates were phenotypically identified as Gram-positive and catalase-negative cocci; and selected for the confirmation of proteolytic activity in skim milk. Also, 34 out of 48 isolates were able to hydrolyze at least one milk protein fraction, indicated by the reduced color intensity of the bands in the SDS-PAGE and also by the appearance of new bands with lower molecular weight compared with the control (see below).

3.2. Screening the proteolytic activity of LABs by SDS-PAGE

The ability of the strains to hydrolyze bovine BLG was determined by the reduced intensity of the bands in the SDS-PAGE analysis. After induction of proteinase activity on milk-citrate-agar media, the bacterial cells were collected at stationary phase of growth and their ability to hydrolyze BLG was assessed at pH 7.0. The hydrolysis of BLG and its degradation by strains was studied by the SDS-PAGE after incubations for 12 and 24 h. The results demonstrated that the strains BMC12C and BMC19H displayed the highest proteolytic activity against BLG. They completely hydrolyzed BLG after incubation for 24 h (Fig. 1). Based on the SDS-PAGE results, the strains BMC12C and BMC19H were considered as the most active strains. Therefore, these strains were selected for further analysis by RP-HPLC.

3.3. Screening the proteolytic activity of LABs by RP-HPLC analysis

RP-HPLC analysis was performed to evaluate the qualitative effects of strains on BLG degradation. RP-HPLC analysis of native and hydrolyzed BLG (after the incubation by BMC12C and BMC19H strains for 24 h) is shown in Fig. 2. The RP-HPLC analysis of BLG proteolysis by BMC12C and BMC19H strains, under optimal conditions of pH and temperature, confirmed the obtained results by the SDS-PAGE. As shown in Fig. 2, the peak representing the native form of BLG disappeared and smaller peaks appeared, suggesting the release of less-hydrophobic peptides. The RP-HPLC profiles demonstrated that BLG was almost

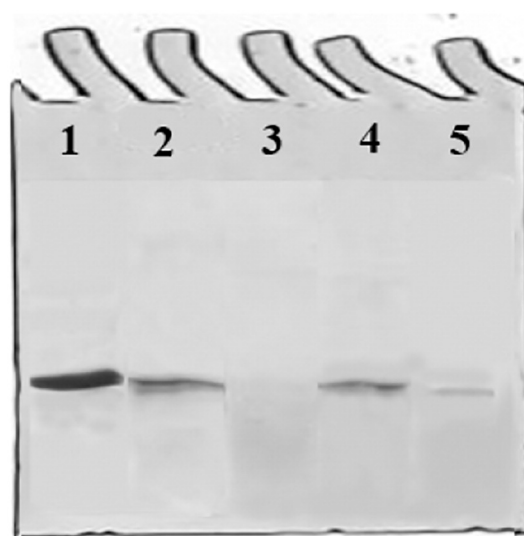


Fig. 1. SDS-PAGE showing the hydrolysis profile of β -lactoglobulin caused by *Lactococcus lactis*. Native BLG (1), BLG hydrolysates resulting from *Lactococcus lactis* BMC12C after 12 h (2) and 24 h (3), and BLG hydrolysates caused by *Lactococcus lactis* BMC19H after 12 h (4) and 24 h (5).

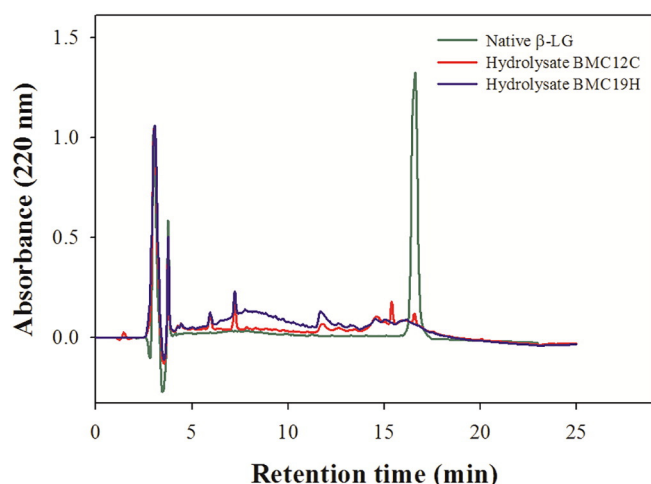


Fig. 2. Reverse phase-HPLC profiles of β -lactoglobulin resulting from hydrolysis of the protein caused by *Lactococcus lactis* BMC12C and *Lactococcus lactis* BMC19H.

completely hydrolyzed due to the strong proteolytic activity of BMC12C and BMC19H strains. The HPLC profiles of BLG hydrolysates in the presence of other studied strains showed that BLG was still present in the treated samples, but the appearance of less-hydrophobic peaks suggested that BLG was partially hydrolyzed (data not shown).

3.4. Response of patient sera to native and hydrolysed BLG by competitive ELISA assay

One purpose of this study is to find out if the BLG hydrolysates (after the incubation by BMC12C and BMC19H strains for 24 h) can be an IgG and/or IgE binding epitope. Therefore, the effect of proteolysis of β -lactoglobulin by *Cocci lactic acid* bacteria on its IgE binding ability was studied using ELISA competitive assay. A pool of IgE containing sera, obtained from 21 CMA patients, was incubated with increasing concentrations of native and hydrolysed BLG (by proteolytic activity of BMC12C and BMC19H) as competitive antigens. The serum antibody/BLG complexes were then added to 96-well plates pre-coated with native BLG. The amount of soluble IgE bound to the plate (not bound to the competitive antigen) was quantified with an enzyme conjugated IgE specific secondary antibody. The competitive ELISA standard curves (Fig. 3) showed that the produced BLG hydrolysates, after the incubation of the native protein by BMC12C and BMC19H strains for 24 h, are about 16 and 25-fold weaker antigens of serum IgE than the native

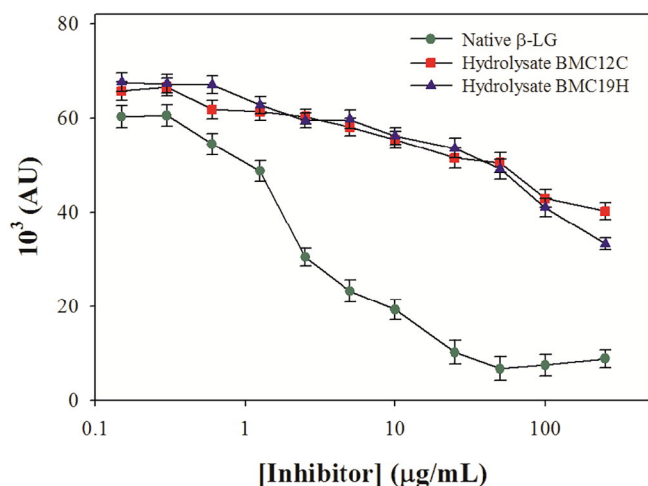


Fig. 3. Inhibition of anti-BLG IgE in serum pool. Comparison between native BLG and hydrolysed BLG caused by *Lactococcus lactis* BMC12C and *Lactococcus lactis* BMC19H.

BLG, respectively. The calculated IC_{50} values for the native BLG, the BLG hydrolysates caused by the BMC12C strain, and the BLG hydrolysates caused by the BMC19H strain were 1.71 ± 0.32 , 27.29 ± 1.08 , and $42.67 \pm 1.96 \mu\text{g/mL}$, respectively (Table 1).

If SDS-PAGE and RP-HPLC show a good degradation of BLG after the incubation with whole cells of BMC12C and BMC19H strains for 24 h, this degradation might only be partial, leaving peptides long enough to bind the antibodies. Herein, from the curves of inhibition on Fig. 3, it can be observed that with the increase of inhibitor concentration, the difference in the IgE binding ability between native and hydrolysed BLGs progressively increased. Finally, the results demonstrated that the hydrolysed BLGs were less recognized than the native BLG by IgE from CMA patients. This may be due to the more effective hydrolysis of milk β -lactoglobulin (by proteolytic activity of BMC12C and BMC19H strains) with higher possibility for cleavage of epitopes.

During fermentation, release of proteases and peptidases results in more effective hydrolysis of milk proteins with higher possibility for cleavage of epitopes. Nevertheless, it is also possible that by further cleavage of the peptides into smaller peptides and amino acids by peptidases, some hidden epitopes or linear epitopes could be exposed. It has been found that at the beginning of the fermentation, antigenicity of BLG decreased gradually but at longer fermentation time it slightly increased, what indicates that there was no linear correlation between the degree of hydrolysis of milk protein and the antigenicity of whey proteins during the fermentation [63]. Ehn et al. used different strains of *L. helveticus* to hydrolyze milk whey proteins and observed 80% hydrolysis of BLG. However, this did not affect the epitope recognition by IgE as judged by the unchanged inhibition pattern (ELISA, human serum) after this treatment. This suggests that the extracellular proteolytic activity in the fermentation process did not completely degrade the IgE epitopes. It is also possible that the degradation was only partial, leaving peptides long enough to bind the antibodies, or it is possible that better access to some buried internal epitopes compensated any hydrolytic reduction of external epitopes. It is also possible that the preheating temperature might have a significant impact on the changes of the antigenicity of whey proteins [69].

However, further in vitro and in vivo studies are necessary to understand better the mechanism of allergenicity of BLG and the methods to decrease it. Additionally, it would be interesting to study the combination of various methods, such as fermentation and hydrolysis with animal and plant proteases, heat treatment, high pressure, and microwave combined with enzymatic proteolysis.

3.5. Identification of the BMC12C and BMC19H strains

Two gram-positive, catalase-negative isolates, which displayed the highest proteolytic activity and greatest reduction in immunoreactivity of bovine β -lactoglobulin, were submitted to amplification and sequencing of 16S rDNA as previously described. Based on the NCBI databases, the partial 16S rDNA sequencing revealed 99% homology to *Lactococcus lactis cremoris* and 94% identity with *Lactococcus lactis hordniae* for the BMC12C and BMC19H strains, respectively.

4. Conclusions

In the present work, we investigated the proteolytic activities of LABs isolated from traditional Iranian dairy products. A collection of

Table 1
Inhibition of IgE binding to native BLG by native protein and hydrolysed BLG caused by *Lactococcus lactis* BMC12C and *Lactococcus lactis* BMC19H.

	Native BLG	Hydrolysed BLG by BMC12C	Hydrolysed BLG by BMC19H
IC_{50} ($\mu\text{g} \cdot \text{mL}^{-1}$)	1.71 ± 0.32	27.29 ± 1.08	42.67 ± 1.96

112 isolates was screened for the production of proteolytic enzymes. According to the ability of these isolates to hydrolyze BLG, 34 isolates were identified with good proteolytic activity against BLG. Further examination of the specific IgE capability to bind the β -lactoglobulin hydrolysates indicated the level of reduction in the immunoreactivity of BLG. The two isolates with the highest proteolytic activity and greatest reduction in the immunoreactivity of bovine BLG were selected and sequenced. 16S rDNA fragment sequencing resulted in the identification of both strains as *Lactococcus lactis*. This strain can reduce the allergenicity of the bovine milk proteins, and therefore, the studied isolates are promising candidates to be applied in the production of fermented hypoallergenic and functional dairy products.

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References

- [1] Z. Li, Y. Luo, L. Feng, Effects of Maillard reaction conditions on the antigenicity of α -lactalbumin and β -lactoglobulin in whey protein conjugated with maltose, *Eur. Food Res. Technol.* 233 (3) (2011) 387–394.
- [2] S.H. Sicherer, H.A. Sampson, Food allergy, *J. Allergy Clin. Immunol.* 125 (2) (2010) S116–S125.
- [3] B.I. Nwaru, L. Hickstein, S.S. Panesar, G. Roberts, A. Muraro, A. Sheikh, Prevalence of common food allergies in Europe: a systematic review and meta-analysis, *Allergy* 69 (8) (2014) 992–1007.
- [4] A. Muraro, T. Werfel, K. Hoffmann-Sommergruber, G. Roberts, K. Beyer, C. Bindslev-Jensen, V. Cardona, A. Dubois, G. Dutoit, P. Eigenmann, EAACI food allergy and anaphylaxis guidelines: diagnosis and management of food allergy, *Allergy* 69 (8) (2014) 1008–1025.
- [5] S.H. Sicherer, H.A. Sampson, Food allergy: epidemiology, pathogenesis, diagnosis, and treatment, *J. Allergy Clin. Immunol.* 133 (2) (2014) 291–307.
- [6] H. Hochwallner, U. Schulmeister, I. Swoboda, S. Spitzauer, R. Valenta, Cow's milk allergy: from allergens to new forms of diagnosis, therapy and prevention, *Methods* 66 (1) (2014) 22–33.
- [7] K. Soares-Weiser, Y. Takwoingi, S.S. Panesar, A. Muraro, T. Werfel, K. Hoffmann-Sommergruber, G. Roberts, S. Halken, L. Poulsen, R. Ree, The diagnosis of food allergy: a systematic review and meta-analysis, *Allergy* 69 (1) (2014) 76–86.
- [8] J.L.A. Marquinez, M.A. Lopez, Food allergy and food poisoning: toxicology on culinary sciences, in: B. Sailas (Ed.), *Examining the Development, Regulation, and Consumption of Functional Foods*, IGI Global 2016, pp. 196–229.
- [9] N. Nicolaou, S. Tsabouri, K.N. Priftis, Reintroduction of cow's milk in milk-allergic children, *Endocr Metab Immune Disord Drug Targets* 14 (1) (2014) 54–62.
- [10] A. Host, S. Halken, Cow's milk allergy: where have we come from and where are we going? *Endocr Metab Immune Disord Drug Targets* 14 (1) (2014) 2–8.
- [11] M. Inam, R.H. Shafique, N. Roohi, M. Irfan, S. Abbas, M. Ismail, Prevalence of sensitization to food allergens and challenge proven food allergy in patients visiting allergy centers in Rawalpindi and Islamabad, Pakistan, *Spring* 5 (1) (2016) 1330.
- [12] S.H. Sicherer, *Food Allergies: A Complete Guide for Eating When Your Life Depends on It*, Johns Hopkins University Press, 2013.
- [13] Y. Vandenplas, J. Marchand, L. Meyns, Symptoms, diagnosis, and treatment of cow's milk allergy, *Curr. Pediatr. Rev.* 11 (4) (2015) 293–297.
- [14] S. Koletzko, B. Niggemann, A. Arató, J.A. Dias, R. Heuschkel, S. Husby, M.L. Mearin, A. Papadopoulou, F.M. Ruemmele, A. Staiano, Diagnostic approach and management of cow's-milk protein allergy in infants and children: ESPGHAN GI Committee practical guidelines, *J. Pediatr. Gastroenterol. Nutr.* 55 (2) (2012) 221–229.
- [15] M. Morisset, C. Aubert-Jacquin, P. Soulaire, D.A. Moneret-Vautrin, C. Dupont, A non-hydrolyzed, fermented milk formula reduces digestive and respiratory events in infants at high risk of allergy, *Eur. J. Clin. Nutr.* 65 (2) (2011) 175–183.
- [16] C. Lifschitz, H. Szajewska, Cow's milk allergy: evidence-based diagnosis and management for the practitioner, *Eur. J. Pediatr.* 174 (2) (2015) 141–150.
- [17] Y.Q. Teng, J.X. Yao, W. Zhu, X. Yan, Q.Y. Lu, J. Jin, Clinical analysis of cow's milk allergy in eleven neonates, *HK J. Paediatr.* 16 (4) (2011) 273–277.
- [18] M. Albenzio, A. Campanozzi, M. D'apolito, A. Santillo, M.P. Mantovani, A. Sevi, Differences in protein fraction from goat and cow milk and their role on cytokine production in children with cow's milk protein allergy, *Small Rumin. Res.* 105 (1) (2012) 202–205.
- [19] P.D. Frederiksen, K.K. Andersen, M. Hammershøj, H.D. Poulsen, J. Sørensen, M. Bakman, K.B. Qvist, L.B. Larsen, Composition and effect of blending of noncoagulating, poorly coagulating, and well-coagulating bovine milk from individual Danish Holstein cows, *J. Dairy Sci.* 94 (10) (2011) 4787–4799.
- [20] R. Lajnaf, L. Picart-Palmade, E. Cases, H. Attia, S. Marchesseau, M.A. Ayadi, The foaming properties of camel and bovine whey: the impact of pH and heat treatment, *Food Chem.* 240 (2018) 295–303.
- [21] R. Jenness, Protein composition of milk, in: H.A. McKenzie (Ed.), *Milk Proteins V1: Chemistry and Molecular Biology*, Academic Press Inc., New York 1970, pp. 17–44.
- [22] Y.-C. Chen, C.-C. Chen, S.-T. Chen, J.-F. Hsieh, Proteomic profiling of the coagulation of milk proteins induced by glucono-delta-lactone, *Food Hydrocoll.* 52 (2016) 137–143.
- [23] R.S. Shoormasti, M.R. Fazlollahi, S. Barzegar, P. Teymourpour, Z. Yazdanyar, Z. Lebaschi, M. Nourizadeh, B. Tazesh, M. Movahedi, H. Kashani, The most common cow's milk allergenic proteins with respect to allergic symptoms in Iranian patients, *Iran. J. Allergy Asthma Immunol.* 15 (2) (2016) 161.
- [24] Y. Vandenplas, E. De Greef, T. Devreker, Treatment of cow's milk protein allergy, *Pediatr. Gastroenterol. Hepatol. Nutr.* 17 (1) (2014) 1–5.
- [25] S. Tsabouri, K. Douros, K.N. Priftis, Cow's milk allergenicity, *Endocr Metab Immune Disord Drug Targets* 14 (1) (2014) 16–26.
- [26] Z. Bartuzi, R.R. Cocco, A. Muraro, A. Nowak-Węgrzyn, Contribution of molecular allergen analysis in diagnosis of milk allergy, *Curr Allergy Asthma Rep* 17 (7) (2017) 46.
- [27] M.Z. Papiz, L. Sawyer, E.E. Eliopoulos, A.C.T. North, J.B.C. Findlay, R. Sivaprasadarao, T.A. Jones, M.E. Newcomer, P.J. Kraulis, The structure of β -lactoglobulin and its similarity to plasma retinol-binding protein, *Nature* 324 (6095) (1986) 383–385.
- [28] A.C. Dave, S.M. Loveday, S.G. Anema, G.B. Jameson, H. Singh, Glycation as a tool to probe the mechanism of β -lactoglobulin nanofibril self-assembly, *J. Agric. Food Chem.* 62 (14) (2014) 3269–3278.
- [29] D. Stanić-Vučinić, T. Čirković-Veličković, The modifications of bovine β -lactoglobulin: effects on its structural and functional properties, *J. Serbian Chem. Soc.* 78 (3) (2013) 443–459.
- [30] H.Y. Çelebioğlu, M. Gudjonsdóttir, S. Meier, J.Ø. Duus, S. Lee, I.S. Chronakis, Spectroscopic studies of the interactions between β -lactoglobulin and bovine submaxillary mucin, *Food Hydrocoll.* 50 (2015) 203–210.
- [31] Z. Teng, R. Xu, Q. Wang, Beta-lactoglobulin-based encapsulating systems as emerging bioavailability enhancers for nutraceuticals: a review, *RSC Adv.* 5 (44) (2015) 35138–35154.
- [32] F. Kimpel, J.J. Schmitt, Milk proteins as nanocarrier systems for hydrophobic nutraceuticals, *J. Food Sci.* 80 (11) (2015) 2361–2366.
- [33] Z. Shafaei, B. Ghalandari, A. Vaseghi, A. Divsalar, T. Haertlé, A.A. Saboury, L. Sawyer, β -Lactoglobulin: an efficient nanocarrier for advanced delivery systems, *Nanomed. Nanotechnol. Biol. Med.* 13 (5) (2017) 1685–1692.
- [34] M.H. Mehraban, R. Yousefi, A. Taheri-Kafrani, F. Panahi, A. Khalafi-Nezhad, Binding study of novel anti-diabetic pyrimidine fused heterocycles to β -lactoglobulin as a carrier protein, *Colloids Surf. B: Biointerfaces* 112 (2013) 374–379.
- [35] W.L. Claeys, S. Cardoen, G. Daube, J. De Block, K. Dewettinck, K. Dierick, L. De Zutter, A. Huyghebaert, H. Imberechts, P. Thiange, Raw or heated cow milk consumption: review of risks and benefits, *Food Control* 31 (1) (2013) 251–262.
- [36] P.D. Dwivedi, M. Das, S. Kumar, A.K. Verma, Safety Assessment of Food Derived from Genetically Modified Crops, in: A. Verma, A. Singh (Eds.), *Animal Biotechnology*, Elsevier 2014, pp. 509–524.
- [37] K.C.M. Verhoeckx, Y.M. Visser, J.L. Baumert, R. Faludi, M. Feys, S. Flanagan, C. Herouet-Guichene, T. Holzhauser, R. Shimojo, N. van der Bolt, Food processing and allergenicity, *Food Chem. Toxicol.* 80 (2015) 223–240.
- [38] C. Dupont, Cow's milk allergy: protein hydrolysates or amino acid formula? in: G. Folkerts, J. Garssen (Eds.), *Pharma-Nutrition*, Springer 2014, pp. 359–371.
- [39] Y. Vandenplas, J. Bhatia, R. Shamir, C. Agostoni, D. Turck, A. Staiano, H. Szajewska, Hydrolyzed formulas for allergy prevention, *J. Pediatr. Gastroenterol. Nutr.* 58 (5) (2014) 549–552.
- [40] G. Bu, Y. Luo, F. Chen, K. Liu, T. Zhu, Milk processing as a tool to reduce cow's milk allergenicity: a mini-review, *Dairy Sci. Technol.* 93 (3) (2013) 211–223.
- [41] A. Fiocchi, L. Dahdah, M. Albarini, A. Martelli, Cow's milk allergy in children and adults, in: M. Ebisawa, B.K. Ballmer-Weber, S. Vieths, R.A. Wood (Eds.), *Food Allergy: Molecular Basis and Clinical Practice*, Karger Publishers 2015, pp. 114–123.
- [42] T. Rahaman, T. Vasiljevic, L. Ramchandran, Conformational changes of β -lactoglobulin induced by shear, heat, and pH—effects on antigenicity, *J. Dairy Sci.* 98 (7) (2015) 4255–4265.
- [43] R. Jiménez-Saiz, S. Benedé, E. Molina, I. López-Expósito, Effect of processing technologies on the allergenicity of food products, *Crit. Rev. Food Sci. Nutr.* 55 (13) (2015) 1902–1917.
- [44] N. Ontiveros, L.K. Flores-Mendoza, V.A. Canizalez-Román, F. Cabrera-Chavez, Food allergy: prevalence and food technology approaches for the control of IgE-mediated food allergy, *J. Nutr. Food Sci.* 2 (5) (2014) 1029–1035.
- [45] U.K. Shandilya, R. Kapila, R.M. Haq, S. Kapila, V.K. Kansal, Effect of thermal processing of cow and buffalo milk on the allergenic response to caseins and whey proteins in mice, *J. Sci. Food Agric.* 93 (9) (2013) 2287–2292.
- [46] C. Bertrand-Harb, A. Baday, M. Dalgalarondo, J.M. Chobert, T. Haertle, Thermal modifications of structure and codenaturation of α -lactalbumin and β -lactoglobulin induce changes of solubility and susceptibility to proteases, *Mol. Nutr. Food Res.* 46 (4) (2002) 283–289.
- [47] L. Robert, J. Labat-Robert, A.-M. Robert, The Maillard reaction. From nutritional problems to preventive medicine, *Pathol. Biol.* 58 (3) (2010) 200–206.
- [48] K.E. El Mecherfi, D. Saidi, O. Kheroua, G. Boudraa, M. Touhami, O. Rouaud, S. Curet, Y. Choiset, H. Rabesona, J.-M. Chobert, Combined microwave and enzymatic treatments for β -lactoglobulin and bovine whey proteins and their effect on the IgE immunoreactivity, *Eur. Food Res. Technol.* 233 (5) (2011) 859.
- [49] A. Taheri-Kafrani, N. Tavakkoli Koupaie, T. Haertlé, β -Lactoglobulin mutant Lys69Asn has attenuated IgE and increased retinol binding activity, *J. Biotechnol.* 212 (2015) 181–188.
- [50] N. Kazem-Farzandi, A. Taheri-Kafrani, T. Haertlé, β -lactoglobulin mutation Ala86Gln improves its ligand binding and reduces its immunoreactivity, *Int. J. Biol. Macromol.* 81 (2015) 340–348.
- [51] M. Yao, Y. Luo, J. Shi, Y. Zhou, Q. Xu, Z. Li, Effects of fermentation by *Lactobacillus rhamnosus* GG on the antigenicity and allergenicity of four cows' milk proteins, *Food Agric. Immunol.* 25 (4) (2014) 545–555.

- [52] J. Shi, Y. Luo, Y. Xiao, Z. Li, Q. Xu, M. Yao, Effects of fermentation by *Lactobacillus casei* on the antigenicity and allergenicity of four bovine milk proteins, *Int. Dairy J.* 35 (1) (2014) 75–80.
- [53] K.R. Elfahri, O.N. Donkor, T. Vasiljevic, Potential of novel *Lactobacillus helveticus* strains and their cell wall bound proteases to release physiologically active peptides from milk proteins, *Int. Dairy J.* 38 (1) (2014) 37–46.
- [54] V. Biscola, F.L. Tulini, Y. Choiset, H. Rabesona, I. Ivanova, J.-M. Chobert, S.D. Todorov, T. Haertlé, B. Franco, Proteolytic activity of *Enterococcus faecalis* VB63F for reduction of allergenicity of bovine milk proteins, *J. Dairy Sci.* 99 (7) (2016) 5144–5154.
- [55] F.L. Tulini, V. Biscola, Y. Choiset, N. Hymery, G.L. Blay, E.C.P. De Martinis, J.-M. Chobert, T. Haertlé, Evaluation of the proteolytic activity of *Enterococcus faecalis* FT132 and *Lactobacillus paracasei* FT700, isolated from dairy products in Brazil, using milk proteins as substrates, *Eur. Food Res. Technol.* 241 (3) (2015) 385–392.
- [56] H.J. Buckenhüske, Selection criteria for lactic acid bacteria to be used as starter cultures for various food commodities, *FEMS Microbiol. Rev.* 12 (1993) 253–271.
- [57] H. Bachmann, D. Molenaar, F. Branco dos Santos, B. Teusink, Experimental evolution and the adjustment of metabolic strategies in lactic acid bacteria, *FEMS Microbiol. Rev.* 41 (2017) S201–S219.
- [58] A. Charcosset, K. Adel-Patient, C. Dupont, H. Bernard, Assessment of IgE and IgG4 binding capacities of cow's milk proteins selectively altered by proteases, *J. Agric. Food Chem.* 64 (2016) 3394–3404.
- [59] K. Babij, J. Bajzert, A. Dąbrowska, M. Sołtysik, A. Zambrowicz, G. Lubec, T. Stefaniak, E. Willak-Janc, J. Chrzanowska, Hydrolysis with *Cucurbita ficifolia* serine protease reduces antigenic response to bovine whey protein concentrate and α s-casein, *Amino Acids* 47 (11) (2015) 2335–2343.
- [60] S. El-Ghaish, A. Ahmadova, I. Hadji-Sfaxi, K.E. El Mecherfi, I. Bazukyan, Y. Choiset, H. Rabesona, M. Sitohy, Y.G. Popov, A.A. Kuliev, F. Mozzi, Potential use of lactic acid bacteria for reduction of allergenicity and for longer conservation of fermented foods, *Trends Food Sci. Technol.* 22 (9) (2011) 509–516.
- [61] S. El-Ghaish, M. Dalgalarrodo, Y. Choiset, M. Sitohy, I. Ivanova, T. Haertlé, J.-M. Chobert, Characterization of a new isolate of *Lactobacillus fermentum* IFO 3956 from Egyptian Ras cheese with proteolytic activity, *Eur. Food Res. Technol.* 230 (4) (2010) 635–643.
- [62] S. El-Ghaish, H. Rabesona, Y. Choiset, M. Sitohy, T. Haertlé, J.-M. Chobert, Proteolysis by *Lactobacillus fermentum* IFO3956 isolated from Egyptian milk products decreases immuno-reactivity of α S1-casein, *J. Dairy Res.* 78 (2) (2011) 203–210.
- [63] G. Bu, Y. Luo, Y. Zhang, F. Chen, Effects of fermentation by lactic acid bacteria on the antigenicity of bovine whey proteins, *J. Sci. Food Agric.* 90 (12) (2010) 2015–2020.
- [64] E. Bernasconi, R. Fritsche, B. Corthesy, Specific effects of denaturation, hydrolysis and exposure to *Lactococcus lactis* on bovine β -lactoglobulin transepithelial transport, antigenicity and allergenicity, *Clin. Exp. Allergy* 36 (6) (2006) 803–814.
- [65] N. Kleber, U. Weyrich, J. Hinrichs, Screening for lactic acid bacteria with potential to reduce antigenic response of β -lactoglobulin in bovine skim milk and sweet whey, *Innovative Food Sci. Emerg. Technol.* 7 (3) (2006) 233–238.
- [66] A. Taheri-Kafrani, Y. Choiset, D.A. Faizullin, Y.F. Zuev, V.V. Bezuglov, J.-M. Chobert, A.-K. Bordbar, T. Haertlé, Interactions of β -lactoglobulin with serotonin and arachidonyl serotonin, *Biopolymers* 95 (12) (2011) 871–880.
- [67] A. Taheri-Kafrani, J.-C. Gaudin, H. Rabesona, C. Nioi, D. Agarwal, M. Drouet, J.-M. Chobert, A.-K. Bordbar, T. Haertlé, Effects of heating and glycation of β -lactoglobulin on its recognition by IgE of sera from cow milk allergy patients, *J. Agric. Food Chem.* 57 (11) (2009) 4974–4982.
- [68] M. Jiang, F. Zhang, C. Wan, Y. Xiong, N.P. Shah, H. Wei, X. Tao, Evaluation of probiotic properties of *Lactobacillus plantarum* WLPL04 isolated from human breast milk, *J. Dairy Sci.* 99 (3) (2016) 1736–1746.
- [69] B.M. Ehn, T. Allmere, E. Telemo, U. Bengtsson, B. Ekstrand, Modification of IgE binding to β -lactoglobulin by fermentation and proteolysis of cow's milk, *J. Agric. Food Chem.* 53 (9) (2005) 3743–3748.