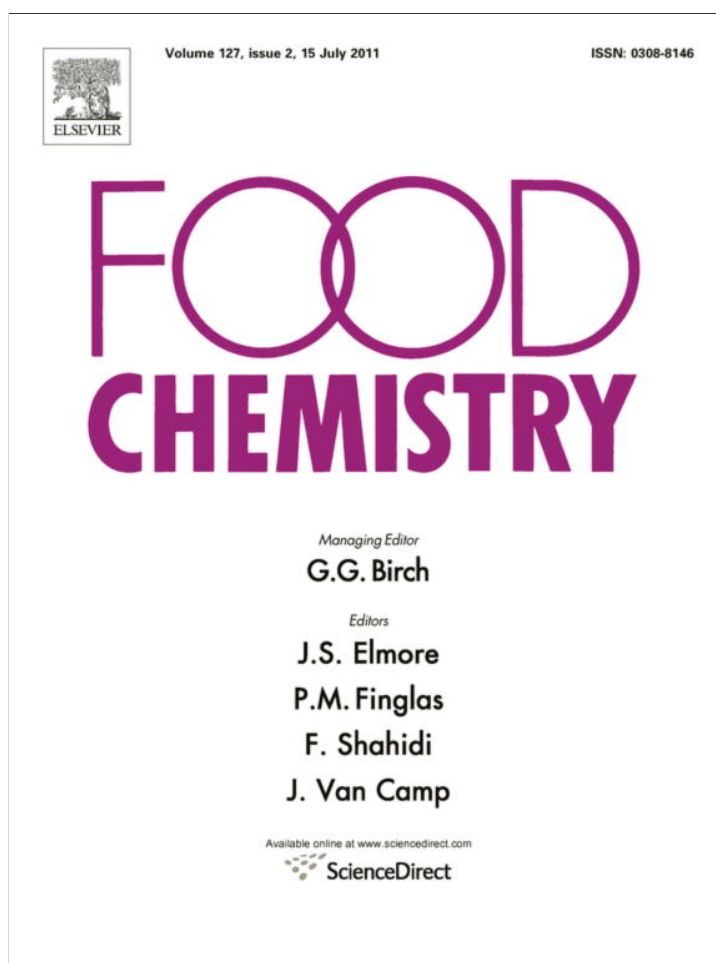


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Proteolytic action of *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 reduces antigenic response to bovine β -lactoglobulin

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ABSTRACT

The whey protein β -lactoglobulin (BLG) is highly allergenic. Lactic acid bacteria can degrade milk proteins. The capacity of *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 to hydrolyse the major BLG epitopes (V41–K60; Y102–R124; L149–I162) and decrease their recognition by IgE of allergic patients was evaluated. The intensity of BLG degradation was analysed by Tricine SDS–PAGE and RP–HPLC. Peptides released were identified by LC–MS/MS and the hydrolysates were tested for their capacity to inhibit IgE binding by ELISA test. *L. delbrueckii* subsp. *bulgaricus* CRL 656 degraded BLG (35%, 8 h). The sequence analysis of the released peptides indicated that this strain degraded three main BLG epitopes. BLG-positive sera (3–5 year old children) were used for testing IgE binding inhibition of BLG hydrolysates from the *Lactobacillus* strain. The hydrolysates were less immuno-reactive (32%) than the heated BLG. *L. delbrueckii* subsp. *bulgaricus* CRL 656 could be used for developing hypoallergenic dairy products.

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1. Introduction

Whey proteins are used as functional food ingredients because of their important nutritional and functional properties (Sinha, Radha, Prakash, & Kaul, 2007). The major whey proteins are β -lactoglobulin (BLG, 55–60%) and α -lactalbumin (ALA, 15–20%); other minor proteins are bovine serum albumin, immunoglobulins, lactoferrin and phospholipoproteins (Peñas, Préstamo, Baeza, Martínez-Molero, & Gomez, 2006).

BLG is absent in human milk and may induce allergies in infants because of the underdevelopment of their gastrointestinal tract and immune system (Pintado, Pintado, & Malcata, 1999). Allergic reactions to bovine milk are favored by rapid absorption of incompletely digested milk proteins caused by the low acidity of infants' gastrointestinal tract and scarce exocrine pancreatic secretion, along with the high buffering capacity of milk. Allergy to cow milk affects 3% of the pediatric population (Hill & Hosking, 1996; Pecquet, Bovetto, Maynard, & Fritsche, 2000); most allergic children have an IgE-mediated allergy as a manifestation of their atopic constitution and only a small group have a cell-mediated allergy with gastro-intestinal symptoms (Kneepkens & Meijer, 2009).

Thus, the study of the IgE responses to native, denatured and hydrolysed milk proteins is critical for the development of new milk derivatives or replacements for sensitive patients.

The BLG monomer is a globular protein composed of 162 amino acids with a molecular weight of 18.3 kDa. The tertiary structure of BLG is rigid, making it highly resistant to gastric digestion (El-Zahar et al., 2005). Hydrolysis of BLG by trypsin/chymotrypsin reduces its allergenicity, but also unmasks hidden allergenic peptides, which have been found to be recognised by specific IgE of allergic patients (Selo et al., 1999). Three tryptic peptides from BLG have been identified as major allergenic epitopes (V41–K60; Y102–R124; L149–I162) (Prioult, Pecquet, & Fliss, 2005). These immuno-reactive structures are distributed rather regularly along the BLG molecule; some of them are short linear sequences, whereas others are large fragments forming conformational epitopes. Therefore, the allergenic potential of milk products depends in great part on their integrity (Peyron, Mouécoucou, Frémont, Sanchez, & Gontard, 2006).

It is very difficult to entirely remove bovine milk or its proteic components from the diet of allergic patients and it is even harder to do so in case of infants who cannot be breast fed. For this reason, the infant formula industry tries to reduce as much as possible the antigenicity of whey proteins (Kim et al., 2007).

Lactic acid bacteria (LAB) are extensively used in the elaboration of fermented foods and more recently, in the development of functional foods. Functional foods are those that can beneficially

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affect one or more target functions in the body, beyond adequate nutritional effects, in a way relevant to an improved state of health and wellbeing and/or reduction of risk of disease (Mollet & Rowland, 2002). In this regard, fermented milk-derived products with reduced allergenic content could be considered as functional foods.

The proteolytic system of LAB, composed of proteinases, peptidases and peptide transport systems, is essential for their growth in milk and milk products (Hébert, Raya, & De Giori, 2000). Proteinases cleave milk proteins into peptides and may therefore have important effects on the further gastro-intestinal milk digestibility, release of the bioactive peptides, and hydrolysis of antigenic epitopes with the consequent decrease of milk allergenicity (Kleber, Weirich, & Hinrichs, 2006). Recently, Bu, Luo, Zhang, and Chen (2010) have shown that the use of combined strains of *Lactobacillus helveticus* and *Streptococcus thermophilus* has a synergic effect on reduction of the antigenicity of whey proteins when grown in skim milk. In previous studies, we showed that other thermophilic LAB strains were able to hydrolyse pure BLG using a non-proliferating cell system (Pescuma, Hébert, Mozzi, & Font de Valdez, 2007; Pescuma et al., 2009). Moreover, *L. acidophilus* CRL 636, *S. thermophilus* CRL 804 and *L. delbrueckii* subsp. *bulgaricus* CRL 656 degraded BLG during their growth in whey protein concentrate (WPC 35%) (Pescuma, Hébert, Mozzi, & Font de Valdez, 2010). Several reports have shown that milk or whey fermentation by LAB could reduce protein allergenicity (Kleber et al., 2006; Bu, Luo, Zhang, & Chen, 2010). However, no studies were carried out using free BLG, avoiding the interference of other proteins or sugars from the food matrix, which can affect the BLG allergenic properties (Davis & Williams, 1998; Svenning, Brynhildsvold, Molland, Langsrud, & Vegarud, 2000; Ehn, Ekstrand, Bengtsson, & Ahlstedt, 2004). Thus, the objective of this study was to evaluate the capacity of *L. delbrueckii* subsp. *bulgaricus* CRL 656 to specifically hydrolyse the BLG allergenic epitopes, and consequently, to reduce the protein recognition by IgE of allergic patients.

2. Materials and methods

2.1. Microorganisms

The strain *L. delbrueckii* subsp. *bulgaricus* CRL 656 used in this work was obtained from the Culture Collection of Centro de Referencia para Lactobacilos (CERELA, San Miguel de Tucumán, Argentina). Cultures were stored at $-20\text{ }^{\circ}\text{C}$ in 10% (w/v) sterile reconstituted skimmed milk containing 0.5% (w/v) yeast extract, 1.0% (w/v) glucose, and 10% (v/v) glycerol. Before use, cultures were propagated twice in MRS (Britania, Buenos Aires, Argentina); 16 h-old active cultures were washed twice with 0.05 M sodium phosphate buffer (pH 7.0) and were inoculated in a chemically defined medium (CDM) as mentioned below.

2.2. Hydrolysis of whey proteins by non-proliferating cells

L. delbrueckii subsp. *bulgaricus* CRL 656 was inoculated in the CDM containing 5 mM CaCl_2 (Hébert et al., 2000) at an initial optical density at 560 nm (OD_{560}) of 0.07 (Spectronic 2000, Bausch, & Lomb, Rochester, NY, USA). The CDM was used to avoid inhibition of LAB proteinase activity by small peptides present in the complex culture media. Cells were harvested by centrifugation (10,000g, $4\text{ }^{\circ}\text{C}$) at the exponential growth phase ($\text{OD}_{560} = 0.65$), washed twice with 0.85% (w/v) saline solution supplemented with 10 mM CaCl_2 , and suspended to a final OD_{560} of 10 in 100 mM sodium phosphate (pH 7.0) with 5 mM CaCl_2 .

Cell suspensions were kept at $37\text{ }^{\circ}\text{C}$ for 30 min for amino acid starvation. *L. delbrueckii* subsp. *bulgaricus* CRL 656 was incubated

with WPC 35% (3.75 mg/ml) (DAVISCO FOODS International Inc., Eden Prairie, MN, USA) or BLG (3 mg/ml) (ICN, Eschwege, Germany) in a cell/protein ratio of 2/1 (v/v). All substrates were previously dissolved in 100 mM sodium phosphate (pH 7.0) supplemented with 5 mM CaCl_2 and heated at $80\text{ }^{\circ}\text{C}$ for 30 min. The mixture (cell-protein) was incubated for 12 h at $37\text{ }^{\circ}\text{C}$ and then centrifuged (10,000g, 10 min, $4\text{ }^{\circ}\text{C}$). Proteins present in the supernatant were analysed by Tricine SDS-PAGE, RP-HPLC and LC-MS/MS, as described before (Pescuma et al., 2007, 2009). The degradation of whey proteins by Tricine SDS-PAGE was evaluated by densitometry analysis of gels by using the QuantiScan software (BIOSOFT 1.5, USA).

2.3. Reversed phase-high performance liquid chromatography (RP-HPLC) analysis of BLG hydrolysates

Briefly, RP-HPLC was performed using an Alliance 2695 system (Waters, Millford, MA, USA). A Symmetry C_{18} column ($3.9 \times 150\text{ mm}$, 300 \AA , $5\text{ }\mu\text{m}$, Waters) was equilibrated with H_2O , trifluoroacetic acid (TFA, 0.055%) solution at a flow rate of 0.15 ml/min. For peptide analysis, a flow rate of 0.15 ml/min, solvent A (0.055% TFA), and solvent B [80% acetonitrile in ultra pure water (v/v) and 0.045% TFA (v/v)] were used. Elution was performed by applying from 0 to 5 min 0% B, then a linear gradient from 5 to 35 min and 100% B from 37 to 40 min. Detection of the peptides was performed between 220 and 330 nm by using a diode array spectrophotometer (model 996, Waters).

2.4. Identification of peptides released from BLG hydrolysates by liquid chromatography-mass spectrometry (LC-MS/MS)

Mass spectrometry analysis was performed in the "Biopolymers-Interaction-Structural Biology" platform located at the INRA Centre of Nantes (INRA Research Unit 1268; (http://www.nantes.inra.fr/plateformes_et_plateaux_techniques/plateforme_bibs)). Samples were reduced or not reduced prior to injection. For reduction, samples obtained as described above were lyophilised, dissolved in a reduction buffer (50 mM Tris, 10 mM dithiothreitol, 6 M guanidinium chloride, pH 8.5), flushed with nitrogen and incubated at $45\text{ }^{\circ}\text{C}$ for 1 h.

Alkylation of free SH-groups was performed by adding 1 M iodoacetamide to a final concentration of 5 mM. After incubating for 30 min at room temperature in the dark, the reaction was stopped by adding TFA to a final concentration of 0.1%. Samples were desalted by solid phase extraction (SPE) on a sep-Pak C_{18} cartridge (Waters). After rinsing the cartridge with a 0.11% TFA (v/v) solution, 5 ml of sample was loaded onto the cartridge and peptides were eluted using an H_2O , acetonitrile, TFA (20/80/0.09, v/v/v) solution. Acetonitrile was evaporated by using a speed vac concentrator (Savant SC 110A, Minnesota, USA).

HPLC was performed on a Waters HPLC system (Waters 616 pump controlled by a Waters 600 controller) coupled to a Finnigan LCQ ion trap spectrophotometer (Finnigan MAT, San Jose, CA). The same column and elution conditions as for RP-HPLC were used for the MS/MS analysis. Spectra were acquired in automated MS/MS mode. The scan rate for MS mode was set between the masses of 400–2000 kDa. Mass data acquisitions were obtained by Mass Lynx Software (Micromass) using automatic switching between MS and MS/MS modes. Peptides eluted from the chromatographic column were detected for 1 s; when their signal reached a defined threshold (4 counts/s) they could be selected for fragmentation. An MS/MS scan (1 s) was then performed on the three most intense peptide ions detected. MS/MS scans of each selected ion were summed until the total fragmentation time attributed to one selected precursor had been reached or the signal in an MS/MS scan had fallen to 0. Acquisitions were performed with the dynamic exclusions of

m/z ratios of already fragmented ions (exclusion of a 70.3 Da mass window around the m/z ratio of previously selected precursors). Fragmentation was performed using argon as the collision gas and the collision energy profile was optimised.

Peptide identification was performed using the Mascot software (version 2.5, Matrix Science) on the MS/MS ion search mode with the following parameters: enzyme: none; peptide mass tolerance: 1 Da; fixed modification: carbamidomethyl (C); variable modification: oxidation (M).

2.5. Immunoglobulin E (IgE) binding capacity of BLG hydrolysates

The IgE binding ability of BLG hydrolysates obtained with *L. delbrueckii* subsp. *bulgaricus* CRL 656 was analysed using competitive enzyme-linked immunosorbent assay (ELISA) test. Native and heat-treated BLG were used as controls. A pool of sera of 14 children (aged 3–5 years old) allergic to milk was used. The sera were previously checked for their specific reactivity to BLG and those with values between 23 and 162 ng/ml of IgE were selected. Sera samples were obtained from the Laboratory of Immunology of Academic Hospital (Angers, France), and their use was approved by the internal Ethical Committee of the hospital.

Micro-titration plates (NUNC Amino 436007, Thermo Scientific, Illkirch, France) were coated with 100 μ l of BLG (5 μ g/ml) in PBS (0.136 M NaCl, 2.68 mM KCl, 1.76 mM KH_2PO_4 , and 10.14 mM $\text{Na}_2\text{HPO}_4 \cdot 12 \text{H}_2\text{O}$), pH 7.4. After three washes with PBS buffer containing 0.1% (v/v) Tween 20 (PBS/T), plates were blocked with 250 μ l of the same buffer and polyvinyl alcohol (PVA, 0.5%, w/v) at 37 °C for 1 h and washed three times with the same buffer. Whilst the blocking was taking place, BLG and the hydrolysate obtained with the *Lactobacillus* strain (dilutions from 1000 to 0.6 μ g/ml) were incubated separately with the selected pool sera (diluted 1/10, v/v) in a ratio 1:1. About 100 μ l of these mixtures (BLG samples and pool of sera) were added to the wells previously coated with BLG. Plates were incubated at 4 °C for 15 h and washed three times with PBS/T. The secondary antibody (Anti-human IgE, Epsilon chain specific, alkaline-phosphatase conjugate developed in goat, 100 μ l, Sigma) was added to the wells; micro plates were further incubated at 37 °C for 2 h and washed three times with PBS/T. Plates were revealed by adding 150 μ l of 4-methylumbelliferyl phosphate (4-MUP, Sigma) diluted 1/5 (v/v) in Tris-HCl pH 9.8 and incubated 90 min at room temperature in the dark. Fluorescence was measured with FL_{x800} plate reader (Biotech Instruments, Inc., Winooski, VT) with a 360 nm excitation filter and a 440 nm emission filter. The inhibition percentages were calculated using the following equation:

$$\left[\frac{(\text{Abs}_0 - \text{Abs}_x)}{(\text{Abs}_0 - \text{Abs}_m)} \right] \times 100$$

where Abs_0 was the absorbance obtained without antigen; Abs_x was the absorbance obtained with different antigen concentrations; Abs_m was the absorbance obtained with the maximum BLG concentration (the minimal absorbance detected).

A curve was made with the obtained values and the protein concentration needed for 50% inhibition (IC_{50}) was calculated from the graph using the Sigma Plot software.

2.6. Statistical analysis

The results shown in Table 1 are mean values and standard deviations of three measurements of two independent assays. Statistical analysis was performed using Minitab 14 software (PA, USA). Comparisons were accomplished by Analysis of Variance (ANOVA) general linear model followed by Tukey's pat-hoc test and $p < 0.05$ was considered significant.

Table 1

Densitometric analysis of BLG and ALA hydrolysates when using separately WPC and BLG as substrates.

WPC			
Time (h)	BLG	ALA	BLG
3	37.6 \pm 4.21 ^a	4.3 \pm 0.16 ^{1b}	23.6 \pm 1.11 ^c
6	40.6 \pm 3.31 ^a	11.8 \pm 2.4 ^{2b}	27.3 \pm 1.8 ^{2c}
8	52.9 \pm 9.02 ^a	18.4 \pm 4.7 ^{3b}	35.2 \pm 0.6 ^{3c}
12	56.7 \pm 0.42 ^a	20.1 \pm 3.1 ^{3b}	37.4 \pm 1.4 ^{3c}

Means in a row or column not sharing the same superscript (letters or numbers, respectively) differed significantly ($p < 0.05$) by a Tukey's pat-hoc test.

3. Results

3.1. Hydrolysis of whey proteins by *L. delbrueckii* subsp. *bulgaricus* CRL 656

L. delbrueckii subsp. *bulgaricus* CRL 656 was able to degrade BLG either alone or in WPC. However, BLG hydrolysis was greater (1.6 times after 12 h incubation) when included in WPC (Table 1). Degradation of whey proteins using WPC showed that BLG was degraded mainly during the first 3 h of incubation, after which the hydrolysis became less intense (Table 1). In contrast, ALA (second main protein in WPC) was degraded gradually during the first 8 h. No significant changes in the hydrolysis percentage were detected after 8 h time point (Table 1). Hydrolysis of pure BLG allowed the visualisation of the derived peptides without interfering bands of other whey proteins present in WPC (Fig. 1). Degradation of pure BLG displayed a similar profile as in WPC. An increase of low molecular weight bands (between 15.6 and 5.6 kDa) was observed when increasing the incubation time. Additional peptides corresponding to bands of 9.2, 13.4 and 14.5 kDa were detected after 12 h of incubation (Fig. 1). Maximal BLG hydrolysis was obtained after 8 h of incubation. This sample was selected for further analysis.

3.2. RP-HPLC analysis

The RP-HPLC graph shows the profiles for native, heated and hydrolysed BLG. BLG was degraded after 8 h of incubation releasing 6 main peaks, three corresponding to hydrophilic peptides and three eluted later showing a more hydrophobic behaviour (pointed with arrows in Fig. 2). Additionally, some smaller size peaks were detected between 18 and 22 min of elution. The

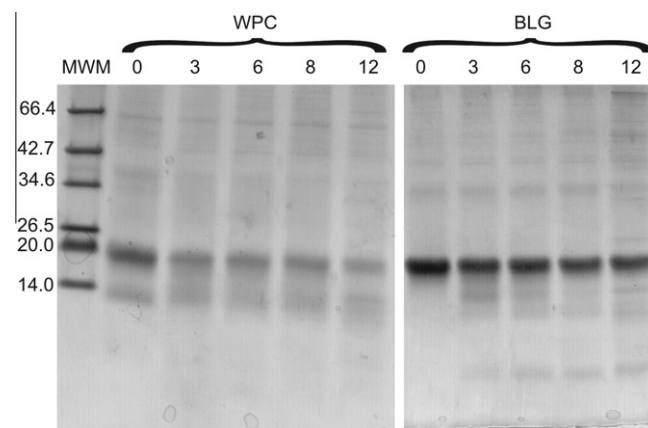


Fig. 1. Whey protein degradation observed by Tricine SDS-PAGE showing ALA and BLG hydrolysis in WPC and BLG degradation in its pure form. A wide range (2–212 kDa) molecular weight marker (MWM) from New England Biolabs was used. Numbers in the Figure correspond to incubation times.

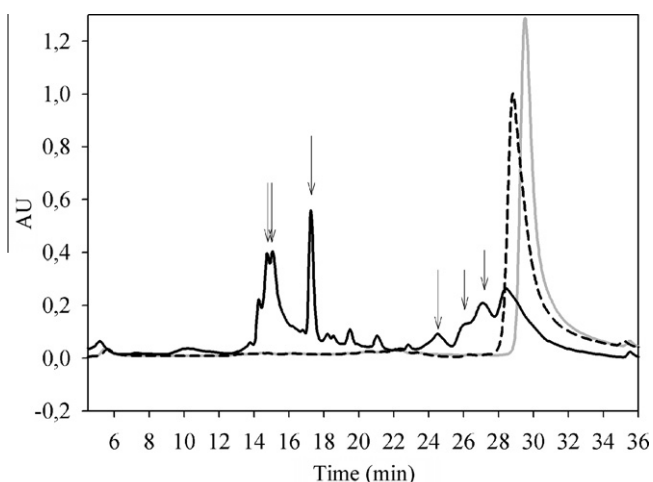


Fig. 2. RP-HPLC chromatograms of native (grey straight line); heated (black dashed line); and hydrolysed BLG by *L. delbrueckii* subsp. *bulgaricus* CRL 656 (black straight line). Arrows show the main peptides. Absorbance was measured at 220 nm.

chromatographic patterns of heated and native BLG differed; the heated protein started to elute 90 s before native BLG, showing a broader peak. The difference in the BLG profile could be due to conformational changes (denaturalisation) of the molecule after the heat treatment.

3.3. Identification of the peptides by LC–MS/MS mass spectrometry

Fourteen peptides of molecular mass ranging between 544.07 and 2895.67 Da were detected by LC–MS/MS in the BLG hydrolysate from *L. delbrueckii* subsp. *bulgaricus* CRL 656 (Table 2). Most of the identified peptides corresponded to the carboxy-terminal part of the molecule, whilst the smallest peptide (GLDI) corresponded to the amino terminal side (Fig. 3). The commercial BLG used in this work was a mixture of variants A and B. In this respect, peptides corresponding to both variants (2541.23 and 2200.05 Da for variants A and B, respectively) were found. Obtained results showed that *L. delbrueckii* subsp. *bulgaricus* CRL 656 released more peptides with the G, T and E amino acids in the C-terminal part of the protein. Interestingly, sequences corresponding to truncated parts of the main allergenic sequences (V41–K60; Y102–R124; L149–I162) of BLG were found in the hydrolysate, the sequence

Table 2
Identification by LC–MS/MS of peptides released from BLG degradation by *L. delbrueckii* subsp. *bulgaricus* CRL 656.

Experimental mass (Da)	Peptide sequence	Theoretical mass (Da)	Allergenic sequence ^a
545.08	K.GLDI.K	544.07	N
562	L.VLDTD.Y	651	N
629	A.QKKII.A	627.99	N
659.02	E.GDLEIL.L	658.02	H
686.16	E.EQCH.I	685.16	H
813.76	E.ELKPTPE.G	812.76	H
727.38	E.ELKPYPEGDLEIL.L	1452.75	H
801.97	L.SFNPTQLLEEQCHI	1601.93	H
848.08	E.ELKPTPEGDLEILLQ.K	1694.15	H
1101.03	P.TPEGDLEILLQKWENDECA.Q	2200.05	H
848.08	L.LFCMENSAPPEQSLACQCLVR.T	2541.23	H
801.97	S.AEPEQSLACQCLVRTPEVDDEA.L	2402.9	H
848.08	E.ELKPTPEGDLEILLQKWENDECA	2541.23	H
1448.84	R.TPEVDDEALEKFDKALKALPMHIRLS	2895.67	H

^a The symbols indicate that the allergenic sequence is: H, hydrolysed; N, not included.

V41–K60 being the most intensively truncated epitope (6 peptides), whilst 5 peptides containing cleaved sequences of the epitope Y102–R124 (2 peptides) and L149–I162 (3 peptides) were detected.

3.4. Allergenicity of BLG hydrolysates

IgE binding inhibition produced by native BLG as determined by ELISA competitive test was observed with minimal concentrations of 5 µg/ml of BLG and increased with concentrations up to 50 µg/ml, after which it remained stable with absorbance values similar to those of the control (non-coated wells) (Fig. 4).

The IgE binding ability to BLG was significantly reduced after heat treatment (Fig. 4) with an IC₅₀ value approximately 5 times greater for heated BLG than for native BLG (15.17 and 72.82 µg/ml, respectively). IgE inhibition was observed with concentrations greater than 50 µg/ml. However, at the maximal antigen concentration used, only a 7% decrease in the IgE binding was detected as compared to the native protein. Hydrolysis of heated BLG by the *Lactobacillus* strain decreased the IgE binding (32%) as compared to non-degraded BLG. An IC₅₀ value of 123 µg/ml and incomplete IgE inhibition were observed at the maximal antigen concentrations.

4. Discussion

LAB are extensively used in the food industry, especially for the production of fermented dairy products. Some probiotic LAB strains, which are known to degrade milk proteins in the intestine, are included in infant formulas for their capacity to induce oral tolerance (Prioult, Pecquet, & Fliss, 2004; Schouten et al., 2009). Moreover, Pessi et al. (2001) showed that *L. rhamnosus* GG was able to degrade caseins, releasing peptides with immune-modulating effects.

In this work, non-proliferating cells of *L. delbrueckii* subsp. *bulgaricus* CRL 656 were able to degrade the whey protein BLG either free or in WPC; a greater hydrolysis percentage being observed using this latter substrate. In this respect, Bertrand-Harb, Baday, Dalgalarondo, Chobert, and Haertlé (2002) showed that the co-denaturation of ALA with BLG, as occurs during heat treatment, increased their aggregation and resulted in complete exposure of BLG peptic cleavage sites. By these means, the accessibility of the *Lactobacillus* proteinase towards the BLG cleavage sites could also be enhanced.

The identified peptides by LC–MS/MS corresponded mainly to the E45–W61 sequence, probably due to a more accessible part of the protein becoming available after heat treatment. In addition, this sequence showed high proportion of glutamic acid, amino acid found in the carboxy-terminal part of most of the released peptides. In previous studies, we observed that the strain *L. acidophilus* CRL 636 also released peptides corresponding to this part of the molecule. However, differences between this strain and *L. delbrueckii* subsp. *bulgaricus* CRL 656 regarding degradation of BLG were found as the strain CRL 636 preferred to hydrolyse the amino-terminal part of the molecule and to cleave peptides with aspartic acid and glycine in their carboxy-terminal part (Pescuma et al., 2009). This is an interesting finding since these strains could be used combined together to boost BLG hydrolysis. More interestingly, the cell wall-associated proteinase of *L. delbrueckii* subsp. *bulgaricus* CRL 656 could breakdown the three main epitopes of BLG without releasing intact allergenic peptides as revealed by LC–MS/MS analysis. In contrast, complete BLG epitopes may be released during tryptic proteolysis. LAB proteinases, unlike trypsin and other proteolytic enzymes, are non-specific and a broad diversity of cleavage site preferences were found in the few available

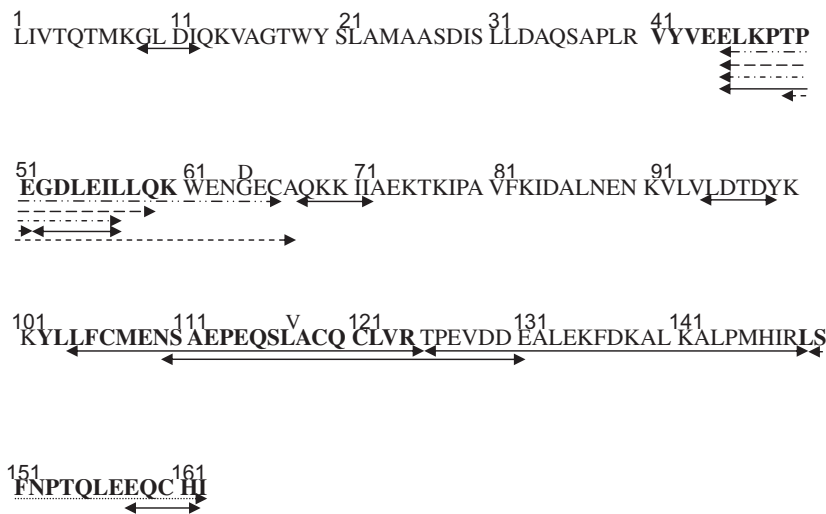


Fig. 3. Location in the primary structure of BLG of the identified peptides (arrows) released by *L. delbrueckii* subsp. *bulgaricus* CRL 656 proteinase. Main epitopes are indicated in bold characters.

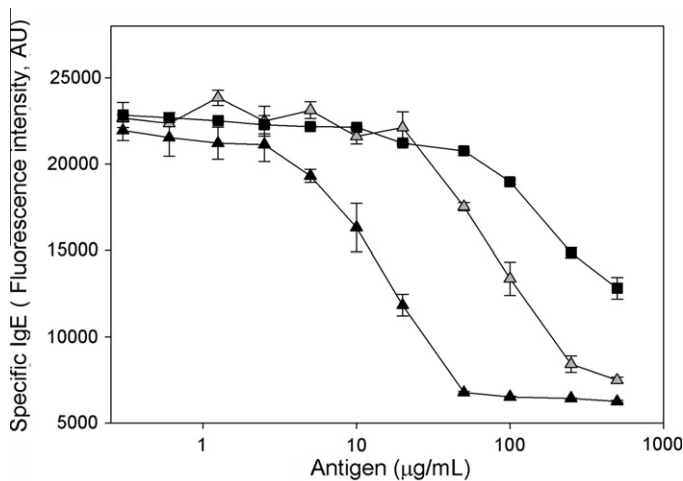


Fig. 4. Inhibition of anti-BLG IgE in the pool of sera using native (black triangle); heated (grey triangle); and hydrolysed BLG (black square) by *L. delbrueckii* subsp. *bulgaricus* CRL 656.

studied (Exterkate, Alting, & Bruinenberg, 1993; Pederson, Mileski, Weimer, & Steele, 1999; Hébert et al., 2008).

Finding new alternatives for formulating hypoallergenic formulae is still an important field since it has been observed that some of the commercial infant formulas are not antigenically neutral and are awaking classic symptoms of cow's milk allergy (Hays, Robert, & Wood, 2005). Moreover, Svenning et al. (2000) showed that a WPC hydrolysate by Colorase PP and Novocin (commercial enzymes) could reduce only 10% BLG allergenicity and that reactivity was not related with peptide size, which is the difference between extensively and partially hydrolysed commercial formulas.

We observed that heat-treatment of BLG reduced its IgE binding ability as previously reported by other authors (Ehn et al., 2004; Peyron et al., 2006). BLG denatures and aggregates at temperatures higher than 70 °C, causing disappearance and masking some conformational and linear epitopes, which explains in part why some patients better tolerate heated dairy products (Taheri-Kafrani et al., 2009). Heated BLG was used to evaluate the capacity of *Lactobacillus delbrueckii* subsp. *bulgaricus* CRL 656 to reduce the IgE binding ability of BLG.

The hydrolysis of BLG by this strain reduced considerably (32%) its recognition by the IgE of allergic children as compared to heated

BLG at the maximum concentration assayed (1 mg/ml). However, degradation of this protein was incomplete and an IgE-binding reaction was still observed. The decrease of allergenicity could be due to hydrolysis as well as to modification of BLG by the *Lactobacillus* proteinases. In this respect, Bernasconi, Fritsché, and Corthésy (2006) showed that incubation of BLG with the proteinase PrtB expressing the *Lactococcus lactis* strain could reduce BLG-IgE recognition, although no hydrolysis was observed. The fact that certain LAB strains could reduce BLG allergenicity without completely degrading the protein could also be of technological interest since it is known that high protein hydrolysis reduces desirable technological characteristics such as aroma, as well as foaming and emulsifying properties, necessary for product acceptance (Sinha et al., 2007; van Beresteijn et al., 1994).

5. Conclusion

In this work we showed for the first time that a *Lactobacillus* cell-envelope proteinase was able to degrade pure BLG and its epitopes *in vitro*, reducing the allergenic response of human sera towards this protein, the major cause of milk allergy in sensitive children.

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