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Effect of Exopolysaccharides on the Hydrolysis of β-Lactoglobulin by *Lactobacillus acidophilus* CRL 636 in an in Vitro Gastric/Pancreatic System

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An analysis of the peptides generated by hydrolysis of BLG by nonproliferating cells of the strain *Lactobacillus acidophilus* CRL 636 was carried out. The effect of polysaccharides (pectin, and two EPS synthesized by two *Streptococcus thermophilus* strains, EPS1190 and EPS804) on BLG digestibility using an in vitro gastric/pancreatic system was analyzed. Polysaccharides are commonly used in the dairy industry to improve food texture; these hydrocolloids may interact with proteins, affecting their digestibility. Nonproliferating cells of *Lb. acidophilus* CRL 636 were able to hydrolyze 52% of BLG. Twenty-six resulting peptides with molecular masses in the range 544−4119 Da were identified by LC-MS/MS. These peptides resulted mostly from the hydrolysis of the more accessible N-terminal part of BLG. Degradation of BLG by pepsin was poor (8%). When BLG was previously hydrolyzed by *Lb. acidophilus* CRL 636, peptic hydrolysis was of 54.8%, while when pectin and EPS1190 were added, hydrolysis was higher (58.2 and 57.2%, respectively). Peptides crossing 8 kDa dialysis membranes after trypsin/chymotrypsin hydrolysis were analyzed by HPSEC. The produced peptides were smaller when BLG was hydrolyzed previously by the *Lb. acidophilus* strain. Moreover, in the presence of pectin, the amount of the larger peptide (3.5 kDa) observed in the size exclusion chromatograms was considerably decreased. Our studies showed that prehydrolysis of BLG by *Lb. acidophilus* CRL 636 had a positive influence on BLG digestibility and that polysaccharides may change the peptide profile yielded by trypsin/chymotrypsin hydrolysis, releasing smaller size peptides, which are known to be less immune-reactive. Moreover, *Lb. acidophilus* CRL 636 was able to hydrolyze the main epitopes (41−60, 102−124, and 149−162) of BLG, reducing its allergenic content.

KEYWORDS: Lactic acid bacteria; proteolysis; exopolysaccharide; β-lactoglobulin; digestive system

INTRODUCTION

The beverage market represents a large and growing industry and whey-based drinks are a new promising emerging market segment because of the nutritional properties of whey proteins. Whey, the main subproduct of the cheese industry, has a low total solid content making the mouth feel of whey-based beverages unattractive in comparison with fermented milk products. Thus, the addition of hydrocolloids or the use of exopolysaccharide (EPS)-producing bacteria is needed to increase its density and viscosity (*1*).

Polysaccharides are widely used in the food industry for their thickening and water holding capacities, improving food perception. Although hydrocolloids may display better texturing properties in foods, they can also reduce protein digestibility, and consequently, modify the bioavailability of amino acids (*2*). This effect is due to interactions between polysaccharides and proteins in the food matrix depending on the environmental pH and ionic strength, ionization and charge densities, and the structure and concentration of different biopolymers (*3*, *4*). The formation of complexes between proteins and polysaccharides can decrease protein digestibility and consequently reduce protein absorption (*5*).

β-Lactoglobulin (BLG), the most important soluble protein in milk products, represents approximately 50% of the whey protein in cow milk and is absent in human milk. BLG has a molecular mass (MM) of 18350 Da and is composed of 162 amino acids. Two disulfide bonds stabilize the structure of this globular

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protein, which exists in monomeric form at pH values below 3.0 or as a dimer above 8.0 (*6*). BLG structure is affected by pH, mineral content of the environment, and temperature. BLG susceptibility to the hydrolysis by digestive and nondigestive enzymes has been demonstrated in vivo and in vitro (*7*). Nondigested BLG or high-MM peptides released from BLG hydrolysis may reach the intestinal mucosa almost intact and may cross the intestinal barrier inducing allergenic reactions (*8*). However, heat treatment and decrease of pH that are frequently used in the food industry to reduce microbial contaminations can denature BLG enhancing its degradation by microbial or digestive enzymes (*9*). Recently, it was observed that nonproliferating cells of *Lactobacillus acidophilus* CRL 636 were able to hydrolyze BLG (*10*).

Lactic acid bacteria (LAB) are extensively used in dairy fermentation. These microorganisms cause a significant degree of proteolysis in multiple fermented dairy products; some of them may also produce EPSs (*11*). LAB are able to synthesize a wide range of EPSs with different composition, size, and structure (*11*). Structural analysis combined with rheological studies revealed that there is considerable variation among the different EPSs. Some of them exhibit remarkable thickening and shear-thinning properties and display high intrinsic viscosities. Certain EPSs from LAB show interesting functional and technological properties; thus, these polysaccharides and the EPS-producing LAB strains are used in the elaboration of diverse natural fermented milks and cheeses to improve texture, replacing the addition of exogenous polysaccharides (*12*).

EPS1190 and EPS804 are both neutral EPSs produced by *Streptococcus thermophilus* CRL 1190 and CRL 804, respectively. Mozzi et al. (*11*) showed that *S. thermophilus* CRL 1190 synthesizes a high-MM (1782 kDa) polysaccharide composed of glucose and galactose (1.0:1.5), while *S. thermophilus* CRL 804 produces a low-MM (95 kDa) polysaccharide composed of galactose and rhamnose (2.5:1.0) when grown under statically free pH conditions in skim milk.

Used as a replacement polysaccharide, pectin, a plant hydrocolloid, is a heteropolysaccharide based on 1,4-linked α-D-glucuronic acid interrupted by L-rhamnose residues with a side chain of neutral sugars; its uronic acid residues confer it a negative charge and an isoelectric point around 2−3 (*13*).

The objectives of this work were to characterize the peptides released during BLG hydrolysis by *Lb. acidophilus* CRL 636 and to study the effect of different polysaccharides (pectin and the bacterial food-grade EPS1190 and EPS804) on BLG digestibility, using an in vitro gastric/pancreatic proteolytic system.

MATERIALS AND METHODS

Microorganisms and Culture Media. The strains *Lb. acidophilus* CRL 636 and *S. thermophilus* CRL 804 and CRL 1190 were obtained from the Culture Collection of Centro de Referencia para Lactobacilos (CERELA, San Miguel de Tucumán, Argentina). Cultures were stored at −20 °C in 10% (w/v) sterile reconstituted skim 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 inoculated in a chemically defined medium (CDM) (*14*) as mentioned below.

Exopolysaccharide Isolation and Characterization. Batch cultures of *S. thermophilus* CRL 804 and CRL 1190 were carried out in a 2.5 L fermentor (New Brunswick Scientific Co., BioFlo IV, Edison, NJ, USA) with a working volume of 1.8 L of reconstituted skim milk (10%, v/v). The temperature was maintained at 37 °C, and the pH was automatically controlled at 6.0 by the addition of 6 N NH_4OH. No air was added, and the agitation rate was set at 100 rpm. The culture medium was inoculated separately with a 2% (v/v) inoculum of a 16 h-old active culture of each strain. Fermentations were allowed to proceed for 16 h, and EPS isolation and purification were performed as described earlier (*13*). Lyophilized, pure EPSs (10 mg/mL) were used for determining the molecular mass (MM) by gel permeation chromatography (GPC) using a Knauer Smartline system equipped with a Waters Ultrahydrogel Linear column (7.8 × 300 mm) that was calibrated with dextran standards (ranging from 4.9×10^6 to 8.0×10^4 Da; Sigma, St. Louis, USA) with 0.1 M $NaNO_3$ as the eluent at a flow rate of 0.6 mL min^{-1}. Polysaccharides were detected with a Knauer Smartline 2600 UV detector.

Hydrolysis of BLG by Nonproliferating Cells. BLG hydrolysis was performed as described previously (*10*). Briefly, *Lb. acidophilus* CRL 636 was inoculated in the CDM containing 5 mM $CaCl_2$ (*14*) 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 the inhibition of LAB proteinase activity by small peptides present in complex culture media such as MRS. Cells were harvested by centrifugation (10000*g*, 4 °C) at the exponential growth phase (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 for 30 min at 37 °C for amino acid starvation and thereafter incubated with pure BLG (ICN, Eschwege, Germany) at a cell/protein ratio of approximately 2:1 (v/v). BLG was previously dissolved in 100 mM sodium phosphate (pH 7.0) supplemented with 5 mM $CaCl_2$ and heated at 80 °C for 30 min. The mixture (cells−protein) was incubated for 8 h at 37 °C and then centrifuged (10000*g*, 10 min, 4 °C). Proteins present in the supernatant were analyzed by Tricine SDS−PAGE as described before (*12*). Briefly, samples were suspended in 5 μL of sample buffer (6.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, and 5% β-mercaptoethanol) and heated at 100 °C for 5 min. Tricine SDS−PAGE were carried out on 17% (w/v) polyacrylamide gels on vertical slab electrophoresis cells (BIO RAD Mini PROTEAN 3 System, Hercules, CA, USA) for 7 h at 15 mA. Silver staining was used to visualize the bands in the gels. The degradation of the whey proteins was evaluated by densitometric analysis of gels by using the QuantiScan software (BIOSOFT 1.5, USA). A part of the culture supernatant was lyophilized for further analysis.

LC/MS/MS Mass Spectrometry of the Peptides Released from BLG Hydrolysis by Nonproliferating Cells of *Lb. acidophilus* CRL 636. Mass spectrometry analyses were performed in the platform Biopolymers-Interaction-Structural Biology located at the INRA Centre of Nantes (INRA Research Unit 1268; http://www.nantes.inra.fr/plateformes_et_plateaux_techniques/plateforme_bibs).

The lyophilized culture supernatant containing hydrolyzed BLG obtained in the above assay was suspended in ultrapure water (12 mg/mL) and desalted by solid phase extraction (SPE) on a sep-Pak C_{18} cartridge (Waters, Milford, MA). After rinsing the cartridge with trifluoroacetic acid (TFA, sequential grade, Sigma; 0.11% v/v in ultrapure water) solution, 5 mL of the sample was loaded into the cartridge, and the peptides were eluted using a water/acetonitrile/TFA (20:80:0.09, v/v/v) solution. The remaining acetonitrile was evaporated with a Speed Vac concentrator (Savant SC 110A, Minnesota, USA), and samples were lyophilized. For peptide reduction, samples were dissolved in a reduction buffer (50 mM Tris, 10 mM dithiotreitol, and 6.0 M guanidinium chloride, pH 8.5), flushed with nitrogen, and incubated for 1 h at 45 °C. Alkylation of free SH was performed by adding 1.0 M iodoacetamide to a final concentration of 5 μM. 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% (v/v). Samples were desalted again by SPE as previously described and lyophilized.

Peptide analysis was done by using high performance liquid chromatography (HPLC) with 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). A Symmetry C_{18} column (2.1 × 150 mm, 300 Å, 5 μm, Waters) was equilibrated with solvent A (H_2O, TFA 0.055%; v/v) at a flow rate of 0.2 mL/min. Elution was performed by applying 100% A from 0 to 5 min, then a linear gradient to 100% B (80%, acetonitrile, 20% water, 0.045%, TFA, v/v/v) from 5 to 35 min, and 100% B from 37 to 40 min. Spectra were acquired in automated MS/MS mode. The scan rate for MS mode was set between the masses of 400 to 2000 kDa. 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). Identification was performed by comparing peptides with variants A and B of BLG.

Preparation of BLG/Polysaccharide Mixtures. Bacterial EPS (EPS1190 and EPS804) and pectin were dissolved each (3 mg/0.2 mL) in 100 mM sodium phosphate (pH 7.0). Dispersions were left overnight at 4 °C to allow complete hydration of the molecules. BLG, previously hydrolyzed by *Lb. acidophilus* CRL 636 and each EPS separately, were blended to obtain 0% and 50% (w/w) of relative polysaccharide concentration; merthiolate (Química Medical, Munro, Buenos Aires, Argentina) was added to all solutions at 0.05 μg/mL (final concentration) to avoid contamination.

Enzymatic Hydrolysis. Hydrolysis of BLG was performed in three steps. In the first one, BLG was degraded (BLG_d) by a nonproliferating cell system mimicking hydrolysis by LAB peptidases during fermentation (as described above). In the second step, BLG was incubated with pepsin as it occurs during gastric digestion. Finally, BLG was hydrolyzed by a mixture of trypsin and chymotrypsin (T/C) in a dialysis bag according to Savoie and Gauthier (*15*). Dialysis bags of 8 kDa MW cutoff were used to assess the potential effect of polysaccharides on the diffusion of high MW peptides through the intestinal barrier as in the case of some allergenic proteins (*2*).

Digestion by Pepsin. Seventy microliters of pepsin (2080 U/mg protein, Sigma) at a concentration of 1 mg/mL dissolved in 0.2 N HCl was added to each 3 mL of BLG/polysaccharide mixture. For simulating the in vivo gastric digestion, the pH of the dispersion was progressively reduced from pH 7.0 to 2.0 within 2 h by adding 80 μL of 1 N HCl every 30 min. The digestion was stopped every hour by adding 0.75 M trichloroacetic acid (TCA). Samples were incubated for 30 min, centrifuged, and 10 μL of supernatant withdrawn for amino acids and peptides analysis by the OPA method (*16*) and Tricine SDS−PAGE.

Pancreatic Digestion in Dialysis Bags. The in vitro total digestion of BLG/polysaccharide mixtures was carried out at 37 °C in dialysis bags according to the hydrolysis method developed by Savoie and Gauthier (*15*). Peptic digestion was done as described previously and was stopped by raising the pH to 8.0 with 2.0 N NaOH. Samples were then transferred to dialysis bags with 8 kDa MW cutoff, and 43 μL of a trypsin (12400 U/mg protein, Sigma) and chymotrypsin (40−50 U/mg protein, ICN, Biomedicals Inc.) mixture (1:2.3, w/w), at a weight concentration of 2.5 mg/mL, was added. Dialysis bags were placed in sealed flasks containing 20 mL of 10 mM sodium phosphate buffer at pH 8.0. Flasks were incubated in a water bath at 37 °C with agitation (30 rpm). Digestion products diffusing through the dialysis bags were collected every 2 h for a 6 h-period, and fractions were analyzed by the OPA method and HPSEC (see below). Retentates were freeze-dried and preserved until further protein analysis by Tricine SDS−PAGE as described above.

Characterization of Peptides by High Pressure Size Exclusion Chromatography (HPSEC). Diffused peptides from 8 kDa MW cutoff dialysis bags were characterized by their MM profile by HPSEC. Samples were desalted with a sep-Pak C_{18} cartridge (Waters), concentrated by a Speed Vac, and suspended in 0.2 mL of acetonitrile/TFA (40:0.1%, v/v) aqueous buffer. The same buffer was used as a running buffer. Samples (20 μL) were separated on a TSK-gel G2500PW XL column (300 × 7.8 mm) (Tosoho, Corp., Japan) using a HPLC system (Knauer Smartline, Berlin, Germany) and a 2600 UV detector (Knauer Smartline) set at 214 nm. Molecular masses were determined by comparison to the following standards: enterocin 35 (4290.85 Da (*17*)), KYYGNGVSCNKKGC (1520.35 Da), KYYGNGVSCNK (1232.38 Da), and IPPL (439 Da (*18*)), all peptides obtained from Biosynthesis Inc. (Lewisville, TX, USA).

Statistical Analysis. All assays were carried out in triplicate, and the results were expressed as mean values with standard deviations. Statistical analyses were performed using MINITAB 14 software (State College, PA, USA). Comparisons were accomplished by ANOVA general linear model followed by Tukey's pat-hoc test, and $P < 0.05$ was considered significant.

RESULTS

EPS Characterization. *S. thermophilus* CRL 804 and CRL 1190 produced 16.4 and 4.2 mg/L of EPS, respectively, under the applied conditions (constant pH 6.0). The MM of the isolated EPS determined by GPC were 1674 and 47 kDa for EPS1190 and EPS804, respectively.

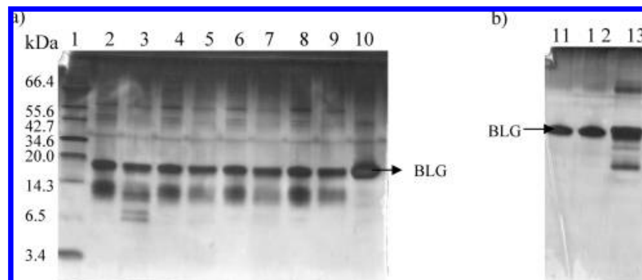

Figure 1. SDS−PAGE showing BLG degradation by pepsin. (**a**) BLG previously degraded by *Lb. acidophilus* CRL 636 (BLG_d) and (**b**) BLG. (1) molecular weight (MW) marker; (2) BLG_d with pectin incubated with pepsin, time 0; (3) BLG_d with pectin incubated with pepsin, time 2 h; (4) BLG_d with no polysaccharide addition incubated with pepsin, time 0; (5) BLG_d with no polysaccharide addition incubated with pepsin, time 2 h; (6) BLG_d with EPS 804 addition incubated with pepsin, time 0; (7), BLG_d with EPS 804 addition incubated with pepsin, time 2 h; (8) BLG_d with EPS 1190 addition incubated with pepsin, time 0; (9) BLG_d with EPS 1190 addition incubated with pepsin, time 2 h; (10) BLG; (11) BLG; (12) BLG incubated with pectin, time 0; and (13) BLG incubated with pepsin for 2 h.

SDS−PAGE and LC/MS/MS Spectrometry Analysis of Peptides Released from BLG Hydrolysis by Nonproliferating Cells of *Lb. acidophilus* CRL 636. Nonproliferating cells of *Lb. acidophilus* CRL 636 were able to degrade 52% of pure BLG after 6 h of incubation (**Figure 1a**; as calculated by densitometric analysis of electrophoretic bands after SDS−PAGE analysis). SDS−PAGE showed the release of peptides with MM between 9.6 and 10.6 kDa. Mass spectrometry analysis also displayed the presence of 26 smaller sized peptides with MM between 495 and 4.119 Da.

Mass spectrometry analysis was performed in two steps: on samples hydrolyzed by *Lb. acidophilus* CRL 636 without any modification to avoid losing small size peptides and on the same hydrolysate desalted by reversed phase after reduction and carboxymethylation to identify peptides linked by disulfide bridges.

Recovery of the sequence after mass spectrometry analysis was not complete. The majority of identified peptides came from the N-terminal sequence (**Table 1** and **Figure 2**). The recovery of the C-terminal sequence was poor (three peptides from the C-terminal part of BLG were observed; **Table 1** and **Figure 2**), and some parts of the BLG sequence were not found. We can observe that BLG was cleaved preferentially after hydrophobic residues since 50% of the peptides released showed hydrophobic amino acids in their amino- or carboxy-terminal part. Only 23% of the peptides showed the presence of aspartic acid in their N-terminal part.

Enzymatic Hydrolysis. Hydrolysis of BLG by pepsin was very limited (1.9%) after 1 h of incubation (pH 5.2). However, the released amino groups increased up to 8.4% after 2 h as measured by the OPA method (**Table 2**). SDS−PAGE showed the presence of peptides of 11−16 kDa obtained from peptic digestion of BLG after 2 h of incubation (**Figure 1b**). The addition of polysaccharides increased the amount of amino groups released from BLG_d up to 3.0, 6.4, and 7.7% with EPS 804, pectin, and EPS1190, respectively (**Table 2**). In the presence of pectin, two additional bands of 5 and 8 kDa were observed by SDS−PAGE (**Figure 1a**), while no important changes in the amount of bands were observed with EPS 804 and EPS 1190.

BLG was not completely digested when it was submitted to intestinal digestion with the T/C mixture since the band corresponding to intact BLG was still observed in the retentates

Table 1. Identification by LC-MS/MS Spectrometry of the Major Peptides Released from BLG Degradation by *Lb. acidophilus* CRL 636

experimental mass (Da)	peptide sequence	theoretical mass (Da)	allergenic sequence[a]
658.35	(52−57) GDLEIL	658.3	H
1570.73	(15−29) VAGTWYSLAMAASDI	1571.69	N
990.51	(22−31) LAMAASDISL	989.48	N
3778.91	(16−50) AGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTP	3763.28	H
3878.84	(89−120) ENKVLVLDTDYKKYLLFCMENSAEPEQSLVCQ	3878.2	H
2713.35	(5−29) QTMKGLDIQKVAGTWYSLAMAASDI	2713.92	N
4433.28	(12−52) IQKVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKPTPEG	4431.55	H
1746.92	(27−42) SDISLLDAQSAPLRVY	1744.93	N
1925.85	(98−112) DYKKYLLFCMENSAE	1927.37	H
3165.63	(20−48) YSLAMAASDISLLDAQSAPLRVYVEELKP	3165.46	H
3755.03	(52−83) GDLEILLQKWENDECAQKKIIAEKTKIPAVFK	3755.89	H
3807.98	(14−48) KVAGTWYSLAMAASDISLLDAQSAPLRVYVEELKP	3808.01	H
4119.16	(1−39) LIVTQTMKGLDIQKVAGTWYSLAMAASDISLLDAQSAPL	4119.06	N
2579.39	(138−159) KALKALPMHIRLSFNPTQLEEQ	2579.14	H
2769.41	(6-31) TMKGLDIQKVAGTWYSLAMAASDISL	2768.66	N
3595.65	(96−124) DTDYKKYLLFCMENSAEPEQSLVCQCLVR	3595.39	C
544.06	(9−13) GLDIQ	544.29	N
559.08	(28−32) DISLL	559.32	N
717.08	(26−32) ASDISLL	717.39	N
857.37	(33−40) DAQSAPLR	856.44	N
899.23	(52−59) GDLEILLQ	899.50	H
1284.65	(29−40) ISLLDAQSAPLR	1282.70	N
637.11	(41−45) VYVEE	637.30	N
673.63	(11−16) DIQKVA	672.38	N
771.08	(50−56) PEGDLEI	771.37	H
1118.78	(33−42) DAQSAPLRVY	1118.60	H

[a] The symbols indicate: H, allergenic sequence hydrolyzed; N, does not include the allergenic sequence; C, includes the allergenic sequence.

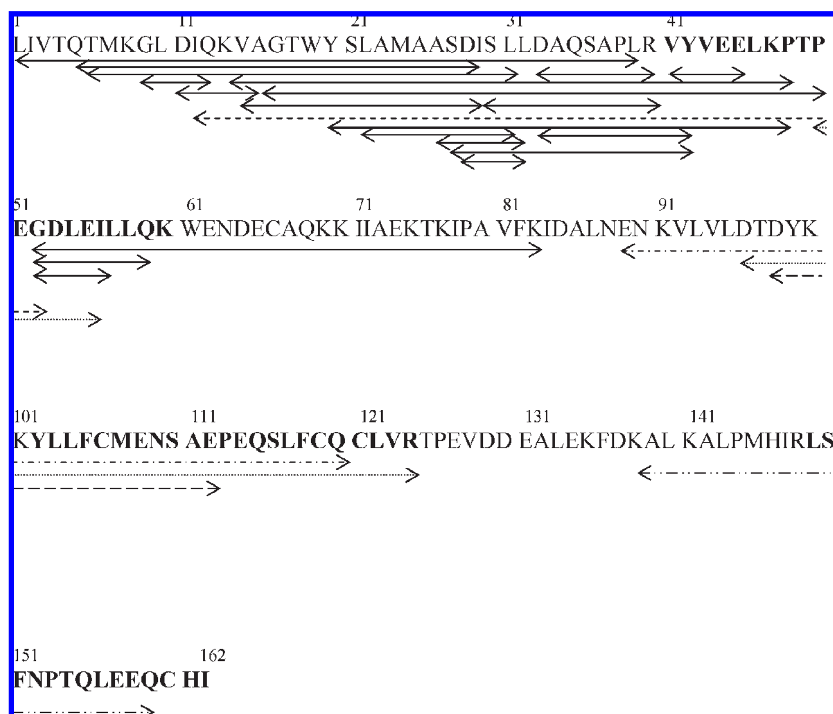

Figure 2. Location in the primary structure of BLG (variant A) of 26 identified peptides (arrows) released by *Lb. acidophilus* CRL 636 proteinase.

(**Figure 3a**). Additionally, bands of 13.0, 11.3, and 6.5 kDa were observed. On the contrary, when BLG was first hydrolyzed by the *Lb. acidophilus* strain (BLG_d) the band corresponding to BLG_d was no longer observed in the retentates, and two main bands of 11.0 and 13.0 kDa were detected, either in the presence or in the absence of polysaccharides.

When samples were submitted to intestinal digestion (with the T/C mixture) and the produced peptides were separated using 8 kDa dialysis bags, an increase in the percentage of released amino groups in the dialysates (5 to 26%, between 2 and 6 h of incubation) was observed all along the incubation period. Only a 3% increase in the amino groups released was observed in BLG_d as compared to that in BLG, while higher increases (6 and 9%) were obtained when EPS 804 or pectin was added to BLG_d (**Figure 4**).

The HPSEC (**Figure 5**) analysis of the dialysates showed the release of lower MM peptides when BLG_d was used as compared to that with native BLG. A high MM peptide (4.2 kDa) was obtained during the digestion of BLG by T/C, while 3.5 kDa was

Table 2. Pepsin Digestion of BLG Previously Degraded by *Lb. acidophilus* CRL 636 in the Presence of Different Types of Polysaccharides

	Percentage of Amino Groups Released[a]				
time (h)	BLG	control[b]	pectin	EPS 804	EPS 1190
0	0 ± 0.1	49.8 ± 15.9	51.8 ± 12.5	49.7 ± 0.5	49.5 ± 0.3
1	3.7 ± 1.9	49.6 ± 15.4	55.9 ± 12.9	50.9 ± 4.5	51.8 ± 7.3
2	8.4 ± 1.8	54.8 ± 10.4	58.2 ± 12.7	52.7 ± 4.3	57.2 ± 0.6

[a] As determined by the OPA method (*20*). [b] BLG previously hydrolyzed by *Lb. acidophilus* CRL 636 without polysaccharide addition.

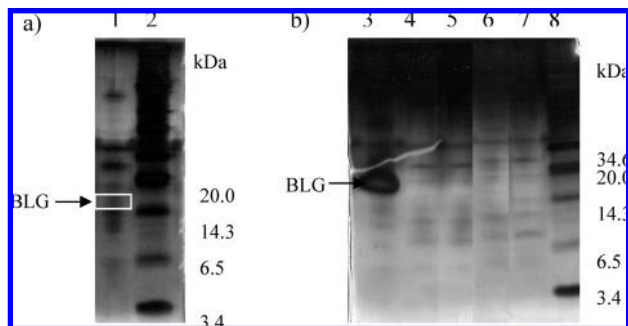

Figure 3. SDS−PAGE showing the retentates in the dialysis bags after 6 h of incubation of (**a**) BLG and (**b**) BLG_d with a mixture of trypsin/chymotrypsin. (1) BLG; (2 and 8) molecular weight marker; (3) nondigested BLG; (4) BLG_d; (5) BLG_d in the presence of pectin; (6) BLG_d in the presence of EPS1190; (7) BLG_d in the presence of EPS 804.

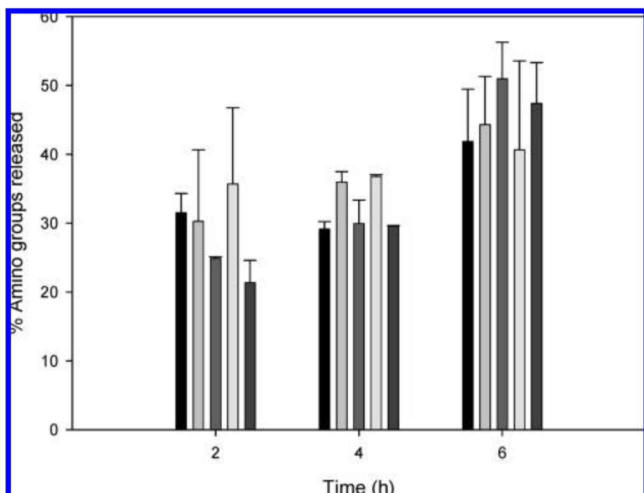

Figure 4. Amino groups release (%) from the 8 kDa dialysis bags after 2, 4, and 6 h incubation. From left to right: BLG, BLG previously digested by *Lb. acidophilus* CRL 636 (BLG_d) without polysaccharide addition, BLG_d with pectin addition, BLG_d with EPS 1190 addition, and BLG_d with EPS 804 addition.

the mass of the largest peptide obtained when BLG_d was hydrolyzed. This peptide was degraded further upon the addition of pectin. Slight differences in the areas of low molecular mass peaks were observed when pectin or EPS 804 were included in the reaction mixtures. In contrast, no difference in the peptide pattern, as compared to that of the control (no polysaccharide addition), was observed when EPS 1190 was added.

DISCUSSION

The effect of LAB on BLG degradation has not been intensively investigated, although a few studies on proteolytic degradation of whey proteins during fermentation have been reported (*19*−*21*)

High resistance of BLG to peptic attack at pH 2.0 was observed before, and the increase of its susceptibility to hydrolysis by heating was also described (*22*). The addition of plant polysaccharides including pectins increased the degree of BLG hydrolysis (*23*). In this work, the effect of pectin and two LAB EPS from *S. thermophilus* strains, commonly used in the industry of dairy fermented products, was analyzed. The MM of the EPS1190 was similar to that observed under static growth conditions (*11*), while the MM of the EPS804 was lower than that reported, probably because of agitation during fermentation.

Slight differences (3.0−7.7%) in BLG hydrolysis were observed when the polysaccharides were added to the reaction mixtures. An increase in BLG degradation by pepsin was observed in the presence of pectin, probably due to electrostatic interactions occurring between BLG (positively charged) and pectin (negatively charged) at pH 2.0 (*2*). Also, an increase in BLG hydrolysis was observed when neutral bacterial EPS was added. Mouécoucou et al. (*8*) showed that a neutral polysaccharide such as xylan was able to interact with BLG; however, no information on the nature of the interaction was provided.

When BLG was degraded by a mixture of trypsin/chymotrypsin in the presence of polysaccharides, the differences observed concerning the amount of each released peptide, as compared to those with samples with no polysaccharides, were greater for peptides with a molecular mass inferior to 400 Da. Moreover, when pectin was added, a decrease in the amount of larger (3.5 kDa) peptides was observed.

The immuno reactivity of milk and whey can be decreased by proteolysis. The techniques lowering the immuno reactivity of proteins include chemical treatments or digestion with proteolytic enzymes, plus high temperatures or high pressure (*9*). Certain LAB demonstrated their lowering effect on the immuno reactivity of BLG (*24*). Prioult et al. (*25*, *26*) showed the ability of strains of *Lactobacilllus paracasei* and *Bifidobacterium lactis* to reduce the antigenicity of BLG after trypsin/chymotrypsin hydrolysis, simulating the intestinal tract.

In this work, a nonproliferating cell system was used to evaluate the hydrolysis of BLG by the cell-wall-associated proteinase of *Lb. acidophilus* CRL 636. No cell lysis was observed during incubation of the nonproliferating cells since neither aminopeptidase activity nor protein content was detected in the supernatants (*10*). BLG was hydrolyzed by a cell-wall-associated proteinase, which showed specificity for α_{s1} and β-casein but not for κ-casein (data not shown); this behavior was similar to that of the P_{III}-type lactococcal proteinases (*27*). Twenty-six peptides issued from BLG degradation by *Lb. acidophilus* were identified. This amount is much higher than the 3 and 10 peptides found by Phromraksa et al. (*28*) and Prioult et al. (*26*) when BLG was hydrolyzed by strains of *Bacillus subtilis* and *Bifidobacterium lactis*, respectively. As our results were not quantitative, it is possible that some peptides are present in very weak amounts in the sample. Although the assays were performed using a mixture of variants A and B of BLG, two of the peptides identified (52−83 and 66−124) matched only for variant A. Braunschweig and Leeb (*29*) showed that variant A was more abundant than variant B in bovine milk. Analysis of the peptides generated during BLG hydrolysis by *Lb. acidophilus* CRL 636 showed the ability of this strain to split BLG at positions 45, 48, 50, 52, 59, 112, 120, and 159 preventing the release of the three intact well-known epitopes (41−60, 102−124, and 149−162) (*26*). *Lb. acidophilus* CRL 636 released the peptide DTDYKKYLLFCMENSAE-PEQSLVCQSLVR (96−124), containing the allergenic sequence

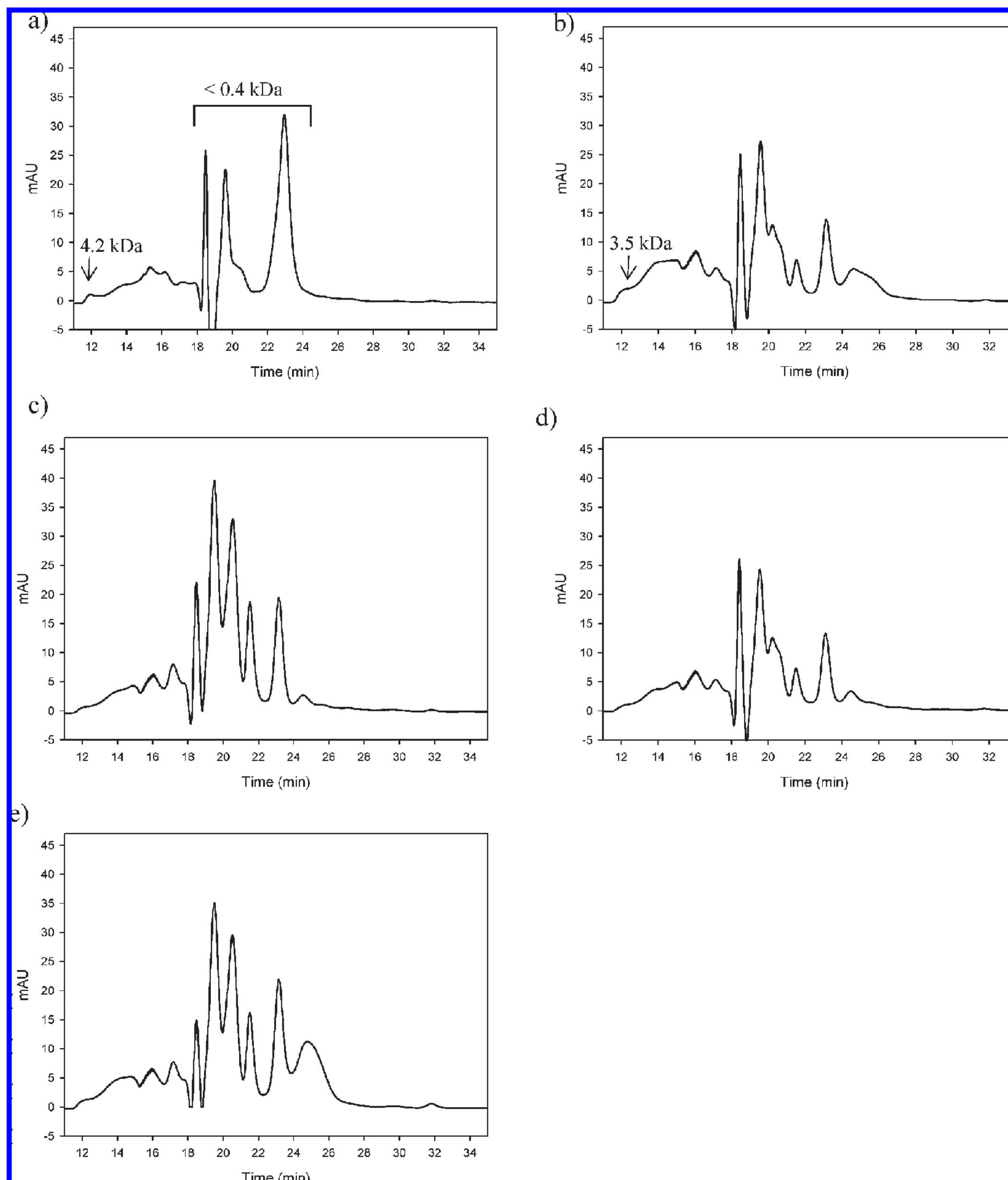

Figure 5. HPSEC profiles of the peptides released from BLG hydrolysis by a mixture of trypsin/chymotrypsin and dialyzed using 8 kDa dialysis bags. (**a**) BLG; (**b**) BLG_d; (**c**) BLG_d in the presence of pectin; (**d**) BLG_d in the presence of EPS1190; (**e**) BLG_d in the presence of EPS 804.

102−124, which is not hydrolyzed by trypsin nor by chymotrypsin. However, this strain is able to hydrolyze BLG at positions 112 and 120 as observed in the sequence of the released peptides 98−112 and 89−120, suggesting that an extensive hydrolysis of BLG by cell proteases could prevent the release of the epitope 102−124. The results showed that *Lb. acidophilus* CRL 636 was able to hydrolyze the main epitopes of BLG; however, peptides had been previously reduced with dithiothreitol for LC-MS/MS. It is well known that disulfide bonds may form large peptide aggregates, which could be recognized by IgE of allergic patients.

It is generally accepted that hydrolysis by trypsin and chymotrypsin decreases the allergenicity of different allergens. Prioult et al. (*26*) observed that the IgE binding capacity of tryptic/chymotryptic peptides of BLG was lower than the intact protein. Indeed, chymotrypsin is able to hydrolyze some of the peptides released by trypsin. This enzyme can hydrolyze the first two amino acids of the peptide VYVEELKPTPEGDLEILLQK (41−60) and the first three amino acids of the peptide LSFNPTQLEEQCHI (149−162), preventing the release of these epitopes.

Interestingly, the capacity of the *Lb. acidophilus* CRL 636 extra cellular proteinase to hydrolyze BLG and especially its epitopes could contribute to preventing allergenic problems frequent in children under 3 years of age due to a poor digestion of BLG. The partial digestion of BLG in children can be explained by the low acidity of the gastrointestinal tract during infancy along with the high buffering capacity of milk, and by the low exocrine pancreatic activity (*30*). Thus, probiotic bacteria are being increasingly used as food supplements in infant formula (*25*) to lower their amounts of allergens.

In addition, *Lb. acidophilus* CRL 636 could also increase BLG digestibility by human digestive proteases since as shown in this work, the amount of peptides with molecular masses smaller than 400 Da was greater when this protein was cleaved before by this LAB strain.

In conclusion, *Lb. acidophilus* CRL 636 could be added as an adjunct culture during milk or whey fermentation in the manufacture of a fermented dairy product with novel and specific properties.

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