# Scavenging of Free Radicals, Antimicrobial, and Cytotoxic Activities of the Maillard Reaction Products of β-Lactoglobulin Glycated with Several Sugars

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The Maillard reaction occurs during many industrial and domestic thermal treatments of foods. It is widely used because of its role in creating colors, flavors, textures, and other functional properties in foodstuffs. Proteins glycated without the use of conventional chemical reagents have improved technofunctional properties such as heat stability, emulsifying, and foaming properties. The present study was carried out to determine the extent to which this reaction can convey antioxidant, antimicrobial, or cytotoxic activities to β-lactoglobulin (BLG) and to its tryptic and peptic hydrolysates. BLG was modified with six different sugars in solution at 60 °C. Antiradical properties were estimated using a radical scavenging activity test. Antimicrobial activities against different bacterial strains were studied with a diffusion disk method. Cytotoxic tests were performed using two cell lines and the 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) rapid colorimetric assay. Glycation induced a radical scavenging activity to BLG, the intensity of which depended on the sugar used for modification. Proteins modified with ribose and arabinose showed the highest radical scavenging activities depicted by about 80 and 60% of 2,2-diphenyl-1-picrylhydrazyl (DPPH) absorption decrease at 515 nm. No antimicrobial effect of any glycated form of BLG against *Escherichia coli*, *Bacillus subtilis*, *Listeria innocua*, and *Streptococcus mutans* was observed. The MTT test showed no enhancement of cytotoxicity by modified proteins and peptides against COS-7 and HL-60 cells. Thus, glycated proteins could be used in formulated food as functional ingredients with a radical scavenging activity able to delay deterioration due to oxidation. This use could be even more advisable considering the lack of toxicity to eukaryotic and prokaryotic cell cultures demonstrated in this work.

**Keywords:** *Free radical scavenger; antimicrobial; cytotoxicity; glycation; β-lactoglobulin*

## INTRODUCTION

The protein fraction of milk is known to contain many valuable components and biologically active substances, which confer special properties for the support of infant development and growth (*1*). Many milk-borne bioactivities are latent, requiring proteolytic release of bioactive peptides from inactive native proteins (*2*). Milk protein-derived bioactive peptides include a variety of substances that are potential modulators of various regulatory processes and reveal multifunctional bioactivities (*3*). Opioid agonist (*4*), opioid antagonist (*5*), inhibitor of angiotensin converting enzyme (ACE) (*6*, *7*), immunomodulator (*8*), antimicrobial (*9*, *10*), and antithrombotic (*11*) activities have been largely described (*12*).

β-Lactoglobulin (BLG) is known to contain an ACE-inhibitory sequence (*13*), but its biological function in milk is still not well-known (*14*).

Because many attempts are made to control food storage and to preserve food from oxidation and microorganism contamination, it is interesting to subject protein to oxidoreductive modification to see whether new biological properties could be induced. The Maillard reaction is one of the major reactions modifying proteins in food and in nature. The examination of how it can influence biological properties of derived proteins and peptides is of particular interest. This reaction, also called nonenzymatic browning or glycation, was first described by the French biochemist Louis Maillard at the beginning of the 20th century (*15*). It corresponds to a spontaneous reaction between amino groups and reducing compounds. In food, it consists of a condensation of the reducing sugars with essentially the ε-amino group of lysyl residues of proteins (*16*). The Maillard reaction occurs frequently during industrial processing, during prolonged storage, and in domestic cooking (*17*), enhancing color, aroma, and flavor. The consequences of this reaction on the biological properties of the modified products have been largely studied on model systems, which consist of heating a single amino acid with a reducing sugar. The Maillard reaction induced (i) antioxidative activity of glucose−glycine (*18*), xylose−lysine (*19*), and xylose−arginine reacting systems (*20*); (ii) antimicrobial activity of xylose−arginine and glucose−histidine systems (*21*−*23*) and of glucose−glycine system (*24*); (iii) cytotoxic activity of glucose−lysine and fructose−lysine systems (*25*); and (iv) clastogenic activity of ribose−lysine (*26*) and glucose−lysine systems (*27*). Proteins modified by the Maillard reaction can also present some of these properties. For example, lysozyme

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modified with dextran by glycation developed a significant antimicrobial activity against both Gram-negative and Gram-positive bacteria (*28*); glycation of casein with glucose or lactose resulted in an enhancement of antioxidant activity when compared with native casein (*29*).

Recent work (*30*) has shown that functional properties, such as thermal stability and emulsifying and foaming properties, of BLG modified by the Maillard reaction are improved, depending on the sugar used during modification. Glycated BLG used as a food ingredient for its functional properties may also decrease the oxidative reactions and/or can influence cellular and microorganism growth.

The aim of this study was to determine how glycation of BLG could induce new biological properties when compared with native protein. Whole glycated BLG and its peptic and tryptic hydrolysates were tested for their free radical scavenging, antimicrobial, and cytotoxic properties.

## MATERIALS AND METHODS

**Protein Purification.** $\beta$-Lactoglobulin (variant A) was purified according to the procedure of Mailliart and Ribadeau Dumas (*31*). The purity of BLG (99%) was assessed by RP-HPLC.

**Reagents.** D-Arabinose, D-galactose, D-glucose, D-lactose, D-rhamnose, D-ribose monohydrates, bovine trypsin TPCK-treated, porcine pepsin, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). *Escherichia coli* HB-101 was from the collection of the Institute of Microbiology, Bulgarian Academy of Science (Sofia, Bulgaria), *Bacillus subtilis* ATCC 6633 was from the American Type Culture Collection (Rockville, MD), *Listeria innocua* F was from the collection of the Ecole Nationale d'Ingénieur des Techniques des Industries Agricoles et Alimentaires (Nantes, France), and *Streptococcus mutans* CIP 103220 T was from the collection of the Institut Pasteur (Paris, France). All other reagents were of analytical grade.

**Achievement of Modified Proteins.** BLG (0.217 mM) and the different sugars (0.217 M) were dissolved in 0.1 M phosphate buffer, pH 6.5. After filtration on an acetate cellulose membrane filter (0.22 $\mu$m diameter, Millipore), mixtures of protein and sugar were put in well-capped flasks and heated in a water bath at 60 °C for 72 h. This mild heat treatment was chosen to limit autoaggregation of BLG. All experiments were performed under strictly anaerobic and sterile conditions; all media were purged and saturated with $N_2$. After heating, the different fractions were dialyzed against distilled water, freeze-dried, and stored at −20 °C. BLG heated without sugar (heated control) was named "heated BLG", and BLG heated in the presence of sugar was named "glycated BLG" or "BLG-sugar".

The quantities of free amino groups were measured using the OPA modified method (see next section). The BLG sequence contains 16 potential reactive amino groups including 1 $\alpha$-$NH_2$ and 15 $\epsilon$-$NH_2$ on lysyl residues. Modified amino groups were deduced from OPA results. According to previous work (*30*, *32*), on average, 11.0, 8.8, 6.7, 6.6, 6.5, and 5.5 amino groups were modified when proteins were heated in the presence of ribose, arabinose, galactose, glucose, rhamnose, and lactose, respectively.

**Determination of Available Amino Groups.** The quantity of available amino groups was determined according to the modified *o*-phthaldialdehyde (OPA) method (*33*). The OPA reagent was prepared daily by mixing 40 mg of OPA, dissolved in 1 mL of methanol, 50 mL of 0.1 M sodium borate buffer, pH 9.3, 100 mg of *N*-dimethyl-2-mercaptoethylammonium chloride (DMMAC), and 1.25 mL of 20% (w/w) SDS in water. Fifty microliters of protein solution (2 mg/mL in 50 mM sodium phosphate buffer, pH 7.8) was added to 1 mL of OPA reagent. The absorbance was read at 340 nm after a minimal delay of 5 min. A calibration curve was obtained by using 0.25−2.00 mM L-leucine as a standard.

**Hydrolysis of BLG.** Native, heated, and glycated BLG (2 mg/mL or 0.11 mM) were dissolved in 0.2 M acetic acid buffer, pH 2.5, for peptic hydrolysis and in 30 mM ammonium carbonate buffer, pH 7.9, for tryptic hydrolysis. Protein samples were dissolved in volatile buffers to allow efficient lyophilization without dialysis after hydrolysis. Porcine pepsin, previously solubilized in distilled water (1 mg/mL), was added to the reaction mixture at an enzyme/substrate (E/S) molar ratio of 1:100. Bovine trypsin, previously solubilized in 0.01 M HCl (1 mg/mL), was added to the reaction mixture at an E/S molar ratio of 1:100. The mixtures were incubated at 37 °C for 120 min. The extent of hydrolysis was controlled by RP-HPLC by measuring the percentage of nonhydrolyzed proteins before freezing at −20 °C, and then hydrolysates were lyophilized.

**Reversed Phase High-Performance Liquid Chromatography (RP-HPLC).** The HPLC equipment consisted of a Waters 2690 separation module system with an integrated solvent, a sample management platform, and a photodiode array detector model 996. The HPLC system was driven with the Millenium32 program (Waters).

Fifty microliters of hydrolyzed and nonhydrolyzed samples was separated by RP-HPLC on a Symmetry 300A $C_{18}$ column (3.9 × 150 mm, Waters) equilibrated in 80% (v/v) solvent A (trifluoroacetic acid/$H_2O$, 0.11:99.89, v/v) and 20% solvent B (acetonitrile/$H_2O$/trifluoroacetic acid, 80:19.91:0.09, v/v/v). Elution was performed with a linear gradient from 80 to 0% solvent A for 30 min. The temperature of the column and solvent was maintained at 30 °C. The flow rate was 0.6 mL/min. Eluted peaks were detected by UV absorbance (214 nm). Peak area corresponding to the nonhydrolyzed form of the protein was integrated. All results were reported to the peak area of the nonhydrolyzed sample that represents 100%.

**Antiradical Activity Tests.** Free radical scavenging activity was measured by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) as stable free radical. Tests solutions of proteins and peptides were dissolved in bidistilled $H_2O$ at different concentrations, and 0.1 mL of each sample was mixed with 0.9 mL of $6 \times 10^{-5}$ M DPPH in methanol solution.

Kinetics experiments were performed on a Cary 1 UV−visible spectrophotometer. Measures were realized every 30 s during 30 min with a constant concentration of proteins of 5 mg/mL. The decrease in absorbance at 515 nm was recorded. After ∼10 min, a plateau was reached. Reaction was considered to be complete after 30 min.

End point measurements were realized with different concentrations of proteins and peptides (varying from 5 to 0.1 mg/mL). The mixture of proteins or peptides with DPPH was left standing at 25 °C for 30 min, and then absorbance at 515 nm was measured.

For all experiments, bidistilled water instead of sample solution was used as control. Gallic acid (60 $\mu$M) was used as positive free radical scavenger control. All experiments were performed in triplicate.

The free radical scavenging activity (R. S. Act.) of a sample was defined as follows:

$$\text{R. S. Act.} = [1 - (\text{Abs}_{515\text{nm}}\ \text{sample}/\text{Abs}_{515\text{nm}}\ \text{blank})] \times 100$$

Inhibitory concentration 50 ($IC_{50}$) and inhibitory concentration 10 ($IC_{10}$) correspond to the concentrations of sample necessary to obtain R. S. Act. values of 50 and 10%, respectively.

**Antibacterial Activity Tests.** Different bacterial strains were used for these tests. *E. coli* and *B. subtilis* were grown on plate count agar (Biokar), *L. innocua* F was grown on Elliker agar (Biokar), and *S. mutans* was grown on Columbia agar (Biokar).

Antibacterial activity was determined according to the disk diffusion method. Protein and peptide samples were dissolved in bidistilled water (1 mg/mL), filtered on 0.22 $\mu$m membranes (Millipore), and stored at 4 °C. Ampicillin (Oxoid) was used as a standard.

Test solutions (10, 25, or 50 μL) were transferred into separate 6 mm diameter paper disks (Whatman No. 1) and left to dry at room temperature under laminar flux. Distilled water was used as a blank.

A preculture of the different strains was realized the day before the manipulation using brain heart infusion (Biokar). Ten microliters of saturated overnight culture was mixed with 10 mL of distilled water and applied at the surface of a Petri dish containing the suitable agar medium. Excess of suspension was eliminated, and dishes were left to dry under laminar flux for 5 min. Paper disks containing proteins or peptides were put at the surface of the inoculated Petri dishes. Finally, Petri dishes were incubated at 37 °C (for *E. coli*, *S. mutans*, and *B. subtilis*) or 30 °C (for *L. innocua*), for 24 or 48 h. Inhibition zones were observed and measured.

**Cytotoxic Activity Tests.** *Cercopithecus aethiops* (monkey, African green) kidney fibroblast COS-7 cells were grown as a monolayer in Dulbecco's Modified Eagle Media (DMEM; Gibco) supplemented with 10% (v/v) fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL). Exponentially growing cultures were maintained in an atmosphere of 5% $CO_2$/95% relative humidity at 37 °C.

Human promyeloblast cells HL-60 were grown in Roswell Park Memorial Institute (RPMI; Biomedia) supplemented with 20% (v/v) fetal calf serum, penicillin (50 units/mL), and streptomycin (50 μg/mL). Exponentially growing cultures were maintained in an atmosphere of 5% $CO_2$/95% relative humidity at 37 °C.

Assays were based on the cleavage of the tetrazolium salt (MTT) into a blue product (formazan) by a mitochondrial enzyme (succinate dehydrogenase) (*34*). The conversion takes place only in living cells, and the amount of formazan produced is proportional to the number of cells present. The assay is based on the method of Edmondson et al. (*35*) with a modification in the protocol of solubilization of formazan, as follows: a solution of 10% (w/v) SDS in 0.01 M HCl was used instead of a solution of 0.04 M HCl in 2-propanol in order to limit the use of toxic chemicals.

Single-cell suspensions were obtained by mechanical deaggregation of the floating cell line HL-60 and by trypsination of the monolayer cell line COS-7. Cell lines (50 μL) were inoculated in 96-well plates at a concentration of 40000 cells/mL (2000 cells/well). Fifty microliters of protein and peptide samples at different concentrations in culture medium was added to each well (columns 4−11). Blanks were realized with 100 μL of culture medium (column 2), and control was obtained with a mixture of 50 μL of cells (40000 cells/mL) and 50 μL of culture medium (column 3). All samples and the control were made in triplicate. After 72 h of incubation at 37 °C, 25 μL of MTT at 2.5 mg/mL in PBS (phosphate-buffered saline) was added to each well. After 4 h of incubation at 37 °C with MTT, 100 μL of 10% (w/v) SDS in 0.01 M HCl was added to each well. After 24 h of incubation at 37 °C, plates were read at 540 nm.

Percentage of cytotoxicity (PC) was calculated as follows:

$$PC\ (\%) = [1 - (Abs_{540nm}\ \text{test wells})/(Abs_{540nm}\ \text{control wells})] \times 100$$

## RESULTS AND DISCUSSION

**Hydrolysis by Pepsin and Trypsin: Determination of the Susceptibility to Hydrolysis of Native, Heated, and Glycated BLG.** Protein samples were hydrolyzed by trypsin and pepsin during 120 min at 37 °C. The quantity of protein left nonhydrolyzed was determined by RP-HPLC. The peak area corresponding to nonhydrolyzed protein was calculated and related to the peak area of the control without addition of enzyme (Table 1). BSA was used as a positive control of hydrolysis.

Native BLG and heated BLG were rapidly hydrolyzed by trypsin. Because glycation sites are located on lysyl and arginyl residues, which are also tryptic cleavage sites, such a modification inhibited tryptic hydrolysis. As expected, and according to previous work (*16*, *32*), glycated BLG resisted to tryptic hydrolysis as a function of its glycation degree. BLG glycated with ribose, which was the most modified sample (11 sites modified of 16 possible), presented 97% of protein left nonhydrolyzed after trypsinolysis. Half the amount of BLG modified with arabinose was still present after trypsinolysis. BLG modified by other sugars exhibited 13−24% of intact proteins after tryptic action, showing a relatively high susceptibility to this protease.

**Table 1. Comparison of Tryptic and Peptic Digestion of Native, Heated, and Glycated BLG and BSA**

| | residual area of nonhydrolyzed protein (%) | |
|---|---|---|
| sample | after peptic hydrolysis | after tryptic hydrolysis |
| native BLG | 95 | 7 |
| heated BLG | 85 | 3 |
| BLG−arabinose | 39 | 48 |
| BLG−galactose | 40 | 19 |
| BLG−glucose | 42 | 17 |
| BLG−lactose | 74 | 13 |
| BLG−rhamnose | 56 | 24 |
| BLG−ribose | 28 | 97 |
| BSA | 0 | 0 |

Peptic hydrolysis gave completely different results. Native BLG is known to resist peptic hydrolysis due to its particular three-dimensional conformation (*36*, *37*). Only 5% of native BLG was hydrolyzed by pepsin in the conditions used. After 72 h of heating at 60 °C, 85% of BLG was left nonhydrolyzed, confirming a high stability of BLG structure after heating. Pepsin susceptibility of BLG depended on its glycation degree (*38*). The most hydrolyzed sample was the ribosylated form of BLG, which showed only 28% of protein left nonhydrolyzed.

**DPPH Test: Effect of Maillard Glycation on the Free Radical Scavenging Activity of BLG.** In these experiments, DPPH in methanol was used as a free radical. This compound is stable and allows evaluation of the antiradical activity of samples. In its radical form, DPPH absorbs at 515 nm, and after its reduction by an antiradical compound, its absorption disappears (*39*). Native, heated, and glycated BLG and their tryptic and peptic hydrolysates were tested for their free radical scavenging activities.

Kinetics experiments were realized first to estimate the time necessary to obtain a relatively stable absorbency of DPPH after being in contact with the protein samples. For all compounds, reaction was very fast in the first 5 min, and then a plateau was rapidly attained. After 30 min, reaction was considered to be achieved and final points were measured.

Radical scavenging activities, $IC_{50}$ and $IC_{10}$, are reported in Figure 1 and Table 2. $IC_{50}$ and $IC_{10}$ of native BLG, heated BLG, and BSA could not be calculated because the highest concentration used (5 mg/mL) was not sufficient to obtain 10% of radical scavenging activity. This is the reason in Table 2, "$>5$ mg/mL" is indicated for $IC_{50}$ and $IC_{10}$ values of these samples. Radical scavenging activities of nonhydrolyzed native proteins and their peptic and tryptic hydrolysates were ∼7%, showing a weak antiradical activity of proteins before glycation.

Nonhydrolyzed BLG modified by the Maillard reaction showed a marked antiradical scavenging activity (Figure 1). Ribosylated BLG showed the highest radical

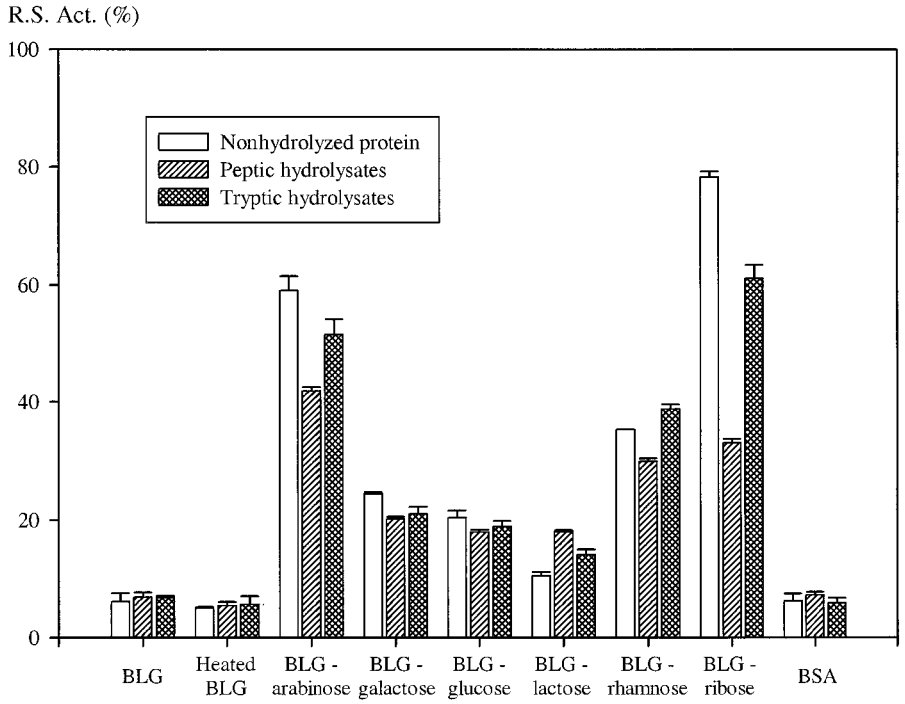


**Figure 1.** Radical scavenging activity (R. S. Act. %) of nonhydrolyzed and peptic and tryptic hydrolysates of native BLG, heated BLG, BLG glycated with arabinose, galactose, glucose, lactose, rhamnose, or ribose, and BSA. Protein concentration was 5 mg/mL. All results were derived from triplicates.

**Table 2. Inhibitory Concentrations of Native, Heated, and Glycated BLG and of BSA against Free Radical of DPPH**

| | proteins | | peptic hydrolysates | | tryptic hydrolysates | |
|---|---|---|---|---|---|---|
| sample | $IC_{10}$ (mg/mL) | $IC_{50}$ (mg/mL) | $IC_{10}$ (mg/mL) | $IC_{50}$ (mg/mL) | $IC_{10}$ (mg/mL) | $IC_{50}$ (mg/mL) |
| native BLG | >5 | >5 | >5 | >5 | >5 | >5 |
| heated BLG | >5 | >5 | >5 | >5 | >5 | >5 |
| BLG−arabinose | 0.9 | 4.0 | 1.4 | >5 | 1.0 | 4.8 |
| BLG−galactose | 2.0 | >5 | 2.5 | >5 | 2.4 | >5 |
| BLG−glucose | 2.1 | >5 | 2.4 | >5 | 2.6 | >5 |
| BLG−lactose | 4.7 | >5 | 2.8 | >5 | 3.5 | >5 |
| BLG−rhamnose | 1.4 | >5 | 1.7 | >5 | 1.3 | >5 |
| BLG−ribose | 0.6 | 2.7 | 1.5 | >5 | 0.8 | 3.8 |
| BSA | >5 | >5 | >5 | >5 | >5 | >5 |

scavenging activity with ~80% of DPPH absorption decrease. BLG modified with arabinose had ~60% of radical scavenging activity. BLG modified with rhamnose, glucose, galactose, and lactose had about 35, 24, 20, and 10% of radical scavenging activity, respectively. These results clearly indicate the role of the Maillard reaction in the free radical scavenging activity of modified proteins, which is in agreement with Nishino et al. (*40*), who showed a radical scavenging activity of milk products prepared by the Maillard reaction.

The free radical scavenging activity of glycated proteins was not directly related to the glycation degree. Although the extent of glycation of BLG modified with rhamnose is about the same as that obtained in the case of glycation with glucose and galactose (see Achievement of Modified Proteins), the rhamnosylated BLG sample exhibited a higher radical scavenging activity than the other two glycated proteins. Formation of melanoidins and heterocycles in the advanced stage of the Maillard reaction (*41*−*43*) could explain the ability of glycated proteins to react with radical compounds (*40*). Nevertheless, BLG modified with rhamnose exhibited a still lower radical scavenging activity than BLG modified with arabinose and ribose, which could be explained by the higher glycation degree obtained with these BLG derivatives.

Free radical scavenging activity of glycated BLG versus the number of moles of reactional primary amino groups, according to the quantity of proteins used during the test and the degree of glycation of each sample, has been measured (Figure 2). This emphasized that activity was also related to the nature of the sugar used for the modification. For example, in the case of BLG glycated with lactose and ribose, the radical scavenging activity corresponding to 150 nmol of reactional primary amino groups was about 10 and 46%, respectively. If radical scavenging activities of samples were related only to glycation degree, for the same amount of reactional primary amino groups, the same activity should be observed, which was not the case in our experiments. According to Figure 2, the nature of the sugar used is another factor influencing the radical scavenging activity of glycated proteins. Such results could be explained, on the one hand, by a more rapid action of ribose during heating, inducing a larger quantity of advanced Maillard products (AMP) as compared with lactose and, on the other hand, by structural and/or chemical differences between sugars, inducing the formation of diversified Maillard reaction products.

According to the results obtained with nonhydrolyzed proteins, modified BLG samples can be ordered from the weakest to the strongest reactant for radical scav-

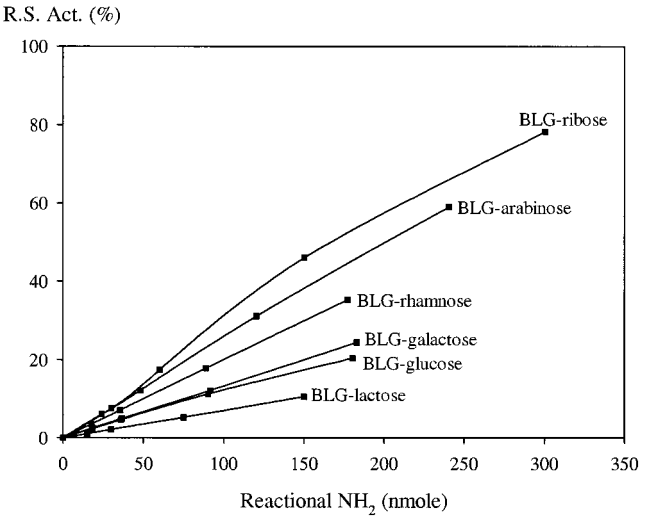


**Figure 2.** Radical scavenging activity (R. S. Act. %) of BLG glycated with arabinose, galactose, glucose, lactose, rhamnose, or ribose as a function of the number of reactional primary amino groups of BLG.

enging activity of DPPH as follows: BLG−lactose < BLG−glucose ≈ BLG−galactose < BLG−rhamnose < BLG−arabinose < BLG−ribose.

The antiradical properties of peptic and tryptic hydrolysates of modified BLG were also studied to assess the influence of hydrolysis on radical scavenging activity of glycated proteins. All proteins did not reach the same extent of hydrolysis as shown by the residual area of nonhydrolyzed proteins (Table 1). Consequently, three parameters could influence radical scavenging activity: glycation degree, nature of the sugar (as was observed for nonhydrolyzed proteins), and hydrolysis degree.

In the case of tryptic hydrolysis, only 25% of proteins remained nonhydrolyzed except for BLG modified with arabinose and ribose. Only minor differences in radical scavenging activity were observed between whole proteins and their tryptic hydrolysates in the case of BLG modified with galactose, glucose, and rhamnose (Figure 1). In contrast, the $IC_{10}$ of BLG modified with lactose decreased by 1.2 mg/mL after tryptic hydrolysis (Table 2), showing a better radical scavenging activity for this sample. Such a result is difficult to explain because, in contrast, the $IC_{10}$ of tryptic hydrolysates of BLG modified with glucose and galactose increased by 0.5 and 0.4 mg/mL, respectively, as compared with nonhydrolyzed proteins. It could be thought that tryptic hydrolysis of BLG modified with lactose exposed new antiradical moieties previously hidden in the core of the globulin molecule. In comparison, $IC_{10}$ and $IC_{50}$ values of BLG modified with arabinose and ribose increased by about 0.1 and 0.2 mg/mL for $IC_{10}$ and by 0.8 and 1.1 mg/mL for $IC_{50}$. BLG modified with arabinose was nearly 50% hydrolyzed by trypsin, whereas BLG modified with ribose was almost not hydrolyzed at all (Table 1). In this case, such activity changes could be explained by additional treatments of these samples during hydrolysis and lyophilization processes as compared with nonhydrolyzed samples.

These observations were even more pronounced when glycated BLG was hydrolyzed with pepsin. In this case, $IC_{10}$ of BLG modified by arabinose and ribose increased by 0.5 and 0.9 mg/mL, respectively, corresponding to a decrease of about 20 and 45% in radical scavenging activity (Figure 1). Other glycated samples did not show such a decrease. Radical scavenging activity of BLG modified with rhamnose, galactose, and glucose decreased by about 5, 4, and 2%, respectively, only. Moreover, an 8% increase of this activity was observed with BLG modified with lactose, representing a decrease by 1.9 mg/mL of its $IC_{10}$.

Nevertheless, even if the radical scavenging activity generally decreased after tryptic or peptic hydrolysis of glycated BLG, it was still higher when compared with that of nonglycated protein.

**Antibiotic Activity: Effect of Glycation of BLG on the Inhibition of Bacterial Growth.** Four different bacteria strains were used to study the antibiotic activity of BLG modified by the Maillard reaction and of its tryptic and peptic hydrolysates. Gram-positive (*B. subtilis* and *S. mutans*) and Gram-negative bacteria (*E. coli* and *L. innocua*) were used to test a variety of morphologically different bacteria.

All of the experiments performed (results not shown) showed that no antibiotic activity against these microorganisms was present in native, heated, or glycated BLG and its hydrolysates.

Einarsson (*21*, *22*) and Einarsson et al. (*23*) observed an inhibition of bacterial growth by Maillard reaction products prepared with arginine−glucose, arginine−xylose, and histidine−glucose model systems. Antimicrobial activity has also been observed in the case of lysozyme modified with dextran by the Maillard reaction (*28*). Nonmodified lysozyme inhibits the growth of only Gram-positive bacteria. Conjugation of dextran to lysozyme resulted in an extension of its antimicrobial spectrum to Gram-negative bacteria. Recently, four bactericidal domains (residues 15−20, 25−40, 78−833, and 92−100) were isolated after tryptic digestion and characterized in the bovine BLG (*44*). In the present study, native BLG did not show any antimicrobial activity with the techniques used. Only the appearance of new antimicrobial activity could be expected. Such an activity is certainly more difficult to induce by a nonspecific chemical modification because of the large spectrum of resistance mechanisms in bacteria.

**Cytotoxic Activity: Effect of Glycation of BLG on the Inhibition of Cellular Growth.** COS-7 and HL-60 cells were used to study the effects of nonhydrolyzed BLG and of tryptic and peptic hydrolysates of BLG modified by the Maillard reaction on cellular growth inhibition. BSA, together with its peptic and tryptic hydrolysates, was used as nonactive control.

Native BLG, heated BLG, BSA, and BLG modified with arabinose, galactose, glucose, lactose, rhamnose, and ribose were used in concentrations ranging from 100 to 0.1 μg/mL. Because BLG modified with arabinose, galactose, lactose, or rhamnose gave very similar results when compared with native or BLG modified with glucose and in order to ensure the clarity of presentation, only the results obtained with native BLG (A), heated BLG (B), BLG glycated by glucose (C) or ribose (D), and BSA (E) are presented in Figure 3.

About 60% of the growth of COS-7 cells was inhibited with nonhydrolyzed BLG at the maximum of protein concentration used (100 μg/mL). The same was observed with heated BLG and BLG glycated with glucose. BLG glycated with ribose and BSA exhibited about 40 and 15% inhibition, respectively. A dose−concentration effect was observed: the smaller the concentration of proteins, the lower the inhibition of cellular growth. The

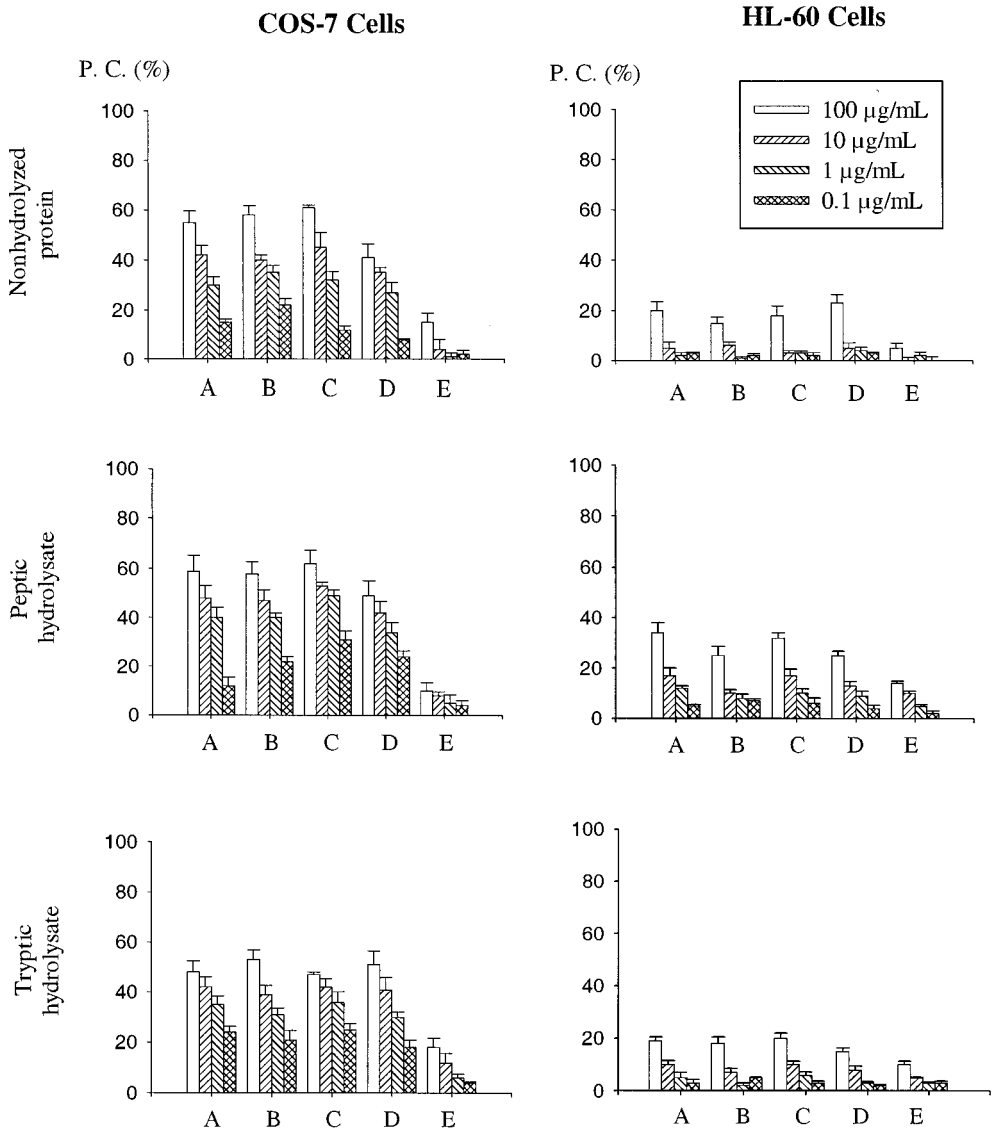


**Figure 3.** Inhibition of growth of COS-7 and HL-60 cells as a function of protein concentration. Nonhydrolyzed and peptic and tryptic hydrolysates of native BLG (A), heated BLG (B), BLG glycated with glucose (C) or ribose (D), and BSA (E) were studied. All results were derived from triplicates.

growth inhibition of COS-7 cells was about the same in the case of peptic and tryptic hydrolysates. Consequently, differences in their inhibiting activity of COS-7 cell growth between native, heated, and glycated BLG did not seem to exist. However, a significant difference was observed between BLG samples and BSA. BLG samples inhibited ~40% more COS-7 cell growth as compared with BSA. However, this certainly could not be related to glycation but rather to the protein used.

Inhibition of HL-60 cell growth with BLG samples was not so efficient as that observed in the case of COS-7 cells. Only 20% of growth inhibition was observed with nonhydrolyzed and tryptic hydrolysates of BLG samples, and ~30−35% of growth inhibition was observed with peptic hydrolysates of BLG samples. Growth inhibition induced by BSA samples was nearly the same in the case of HL-60 cells when compared with COS-7 cells. In the case of tryptic hydrolysates of BLG samples and BSA, quite similar growth inhibitions were observed, showing the very low activity of tryptic hydrolysates of BLG samples. BLG and its peptic hydrolysates showed higher activity when compared with BSA samples.

COS-7 cell growth inhibition might reasonably be related to the cell adhesion because nonadherent HL-60 cells were less inhibited than COS-7 cells. No specific mechanism can be involved in growth inhibition because of too high concentrations used during the experiment. Only a nonspecific inhibition, due to an excess of proteins or peptides, is likely at the origin of such a low inhibition. However, a question can be raised: why was such an activity present only in the case of BLG samples and not in the case of BSA samples? Physicochemical characteristics of BLG such as its amphiphilic properties or its isoelectric point might be involved in the higher activity of BLG as compared to BSA. Meanwhile, no inhibitory activity was induced by glycation whatever. All activities observed were nearly identical in the cases of native, heated, and glycated BLG.

Maillard reactions are known to produce mutagenic, DNA-damaging, and cytotoxic substances especially during food processing and cooking (*45*). This certainly concerns AMP, the so-called advanced Maillard products. Heterocyclic amines are the most potent mutagens known to be formed during the heat processing of food

(*41*). Such compounds should not be formed (at least in detectable quantity) under the conditions used in the present study because of the mild heat treatment applied (72 h at 60 °C). Consequently, in the case of earlier studied products, some innocuousness can be expected.

## ABBREVIATIONS USED

ACE, angiotensin converting enzyme; BLG, β-lactoglobulin; DMEM, Dulbecco's Modified Eagle Media; DPPH, 2,2-diphenyl-1-picrylhydrazyl; MTT, 3-(4,5-dimethylthiazoyl-2-yl) 2,5-diphenyltetrazolium bromide; OPA, orthophthalaldehyde; PBS, phosphate-buffered saline; RPMI, Roswell Park Memorial Institute.

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