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ARTICLE

Biorecognition of Chemically Modified Bovine Serum Albumin with Lactose Prepared under Different Conditions

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Glycoconjugates consist of glycans attached to proteins or lipids. Glycans are involved in important biological functions such as trafficking of glycoconjugates, mediation, and modulation of cell adhesion and signaling. This study was conducted to obtain neoglycoconjugates containing a large number of carbohydrates, added through the condensation of reducing sugars with protein amino groups, whose structures were recognized by lectins. Neoglycoconjugates (BSA-Lac) of bovine serum albumin (BSA) with D-lactose were obtained using two sets of glycation conditions, each previously selected by its ability to glycate proteins extensively. The conditions included dry heat at 60 °C (for 7, 14, 21, and 28 days) and wet heat in 43% relative humidity (RH) at 50 °C (for 5, 10, 15, and 20 h). Products were characterized by gel electrophoresis, tryptophan fluorescence emission spectra, mass spectrometry, free amino group analysis, and their biological recognition established by a galactose-specific lectin and *Escherichia coli* K88 adhesins. BSA-Lac when compared to BSA revealed an increase in monomer mass due to addition of either 13 (dry heat) or 14 (wet heat) lactoses and formation of polymers (in wet conditions). All BSA-Lac products showed reduced intensity of intrinsic fluorescence, decreased amino groups' availability, and were recognized by *Ricinus communis* I lectin (RCAI) and by *E. coli* K88 adhesins. Overall, glycation using both conditions was time-dependent, but greater biorecognition was observed with wet-heat products, due to a higher global glycation and/or to the carbohydrate accessibility. The strategy used in this work represents a simple procedure to obtain glycoconjugates that could be used for recognition studies in biological systems.

KEYWORDS: biorecognition; glycated bovine serum albumin

INTRODUCTION

Carbohydrates attached to proteins and lipids are involved in important processes that influence their structure and function. Glycans also affect intercellular recognition, mediation, and modulation of cell adhesion and signaling, as reported in infection, cancer, and immune responses (*1*).

In nature, the linkage between carbohydrates and proteins is designed as N- or O-links. The *N*-glycosyl linkages are to asparagines, while *O*-glycosyl linkages are to serine, threonine, hydroxylysine, or hydroxyproline (*2*). The limited quantities and heterogeneity of oligosaccharides obtained from glycoproteins often make these molecules unavailable for studies related to define their biological importance.

Alternatively, enzyme-mediated or chemical synthesis of glycoconjugates could provide homogeneous quantities of glycans needed for such studies (*3*). The attractiveness of enzyme-mediated synthesis is the lack of a need for group protection and that glycosidic linkages produced are stereochemically defined (*4*); however, many of these enzymes are not commercially available or in adequate amounts to produce milligrams of oligosaccharides; in addition, although sugar nucleotide substrates are now available, they are expensive (*5*). On the contrary, chemical synthesis of complex carbohydrates involves the coupling of fully protected glycosyl donors groups, leaving its anomeric center with a suitable unprotected glycosyl acceptor that often contains only one free hydroxyl group (*5*). In most cases, these reactions lead to a mixture of two stereoisomers that differ in the configuration of the anomeric center (*6*). An exception to the former is protein glycation or Maillard reaction, a nonenzymatic reaction involving the coupling of protein amino groups to reducing sugars (*7*). The reaction is driven by the sugar carbonyl group that interacts with the nucleophilic amino group of amino acid side chains, producing N-substituted glycosylamine and water (*7*). Protein groups particularly prone to glycation are the terminal amino and side chains of lysine (*8*) and arginine (*9*). For instance, during BSA glycation with lactose, the carbohydrate is bound through the glucose residue (reducing end), leaving a β-galactose available for biorecognition (*10*). Efficiency of glycation varies with reaction conditions. Glycation under

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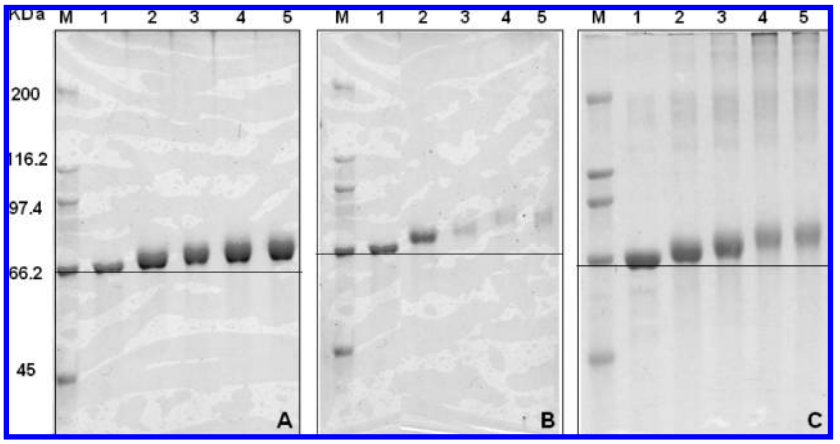

Figure 1. SDS−PAGE in 8% gel. Profiles of untreated (lane 1) and glycated BSA (lanes 2−5). BSA was incubated with lactose at (**A**) 60 °C for 7, 14, 21, and 28 days (lanes 2−5) or (**B**,**C**) under 43% relative humidity and 50 °C for 5, 10, 15, and 20 h (lanes 2−5). Protein loading was 4 μg for gels A and B, and 8 μg for gel C. M: molecular weight markers.

dry-heat (60 °C) conditions leads to the conjugation of only one lactose to BSA (*11*), while the addition of three and four lactoses to porcine albumin was observed after incubation at 43% relative humidity and 60 °C (*12*). Lactosylated proteins were recognized by plant lectins (*11*, *12*).

Lectins are sugar-binding proteins which are highly specific for their sugar moieties. Free or membrane-bound lectins and carbohydrates play important roles in the biological recognition phenomena; that is, membrane-bound lectins in human colon adenocarcinoma cells recognize and internalize neoglycoproteins bearing either α-galactose, α-glucose, or *N*-acetyl-*β*-glucosamine (*13*). Also, in some bacterial illness, blocking the pathogen binding with specific neoglycans has the potential to protect animals against infections (*12*, *14*).

Porcine neonatal and postweaning diarrhea caused by enterotoxigenic *Escherichia coli* (ETEC) results in significant morbidity and mortality, which is associated with large economic losses in the swine industry (*15*, *16*). The most common adhesins of ETEC include K88 (F4), K99 (F5), 987P (F6), F18, and F41 (*17*−*21*). *E. coli* K88 adhesins preferentially bind to glycosphingolipids containing a linked *N*-acetylhexosamine (either *N*-acetyl-*β*-glucosamine or *N*-acetylgalactosamine) or galactose at the terminal position (*22*). Recently, our group has reported that neoglycoconjugates, containing lactosylated albumin, can partially inhibit the adhesion of *E. coli* K88 to mucins, its natural receptor (*12*).

However, improved conditions are required that allow the conjugation of a greater number of and more complex carbohydrates to protein molecules. In this study, two sets of conditions are reported to obtain bovine serum albumin containing 13 and 14 lactoses added by means of nonenzymatic glycation reaction.

MATERIALS AND METHODS

Materials. All reagents used were analytical grade. Bovine serum albumin (BSA), D-lactose (Lac), *ortho*-phthaldialdehyde (OPA), 2-mercaptoethanol, streptavidin peroxidase, glutaraldehyde solution and biotinamidocaproate *n*-hydroxysuccinimide ester, *o*-phenylenediamine dihydrochloride tablet sets (OPD), and Coomassie brilliant blue R were purchased from Sigma-Aldrich (St Louis, MO). Broad range markers were from BIORAD (Hercules, CA), and biotin-labeled *Ricinus communis* I lectin (RCAI) was acquired from Vector (Burlingame, CA) and BSA-Galα(1−3)Gal from Glycorex (Lund, Sweden).

Glycation of BSA in Dry-Heat Conditions. Glycation treatments were conducted according to Kanska and Boratyński (*23*) with some modifications. Preliminary studies show that best conditions involve maintaining molar ratios at 1:780 protein to sugar, pH 8.0, and temperature at 60 °C (data not shown). Briefly, 150 μL of BSA (20 mg/mL) was mixed with 150 μL of sugar solution (80 mg/mL lactose) and 150 μL of 0.1 M phosphate buffer pH 8.0. Samples were frozen at −40 °C, freeze-dried, and later heated at 60 °C for 7, 14, 21, and 28 days. After incubation, samples were dissolved in 300 μL of water, dialyzed to remove salts and free sugar, and kept frozen at −40 °C until use. All experiments were done in duplicate.

Glycation of BSA in Wet-Heat Conditions. In preliminary experiments (data not shown), results showed that glycation can be accelerated by dissolving BSA (100 mg) and lactose (30 mg) in 4 mL of 50 mM carbonate buffer pH 9.0 to reach a molar ratio of approximately 1:60 of protein to sugar. Solutions were lyophilized and later incubated at 43% relative humidity and 50 °C for 5, 10, 15, and 20 h. Humidity was controlled in a desiccator containing a saturated K_2CO_3 solution. After incubation, samples were dissolved in 5 mL of water, extensively dialyzed, freeze-dried, and stored at −40 °C until analysis. Experiments were done in duplicate.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) in 8% acrylamide gels was performed under reducing conditions (*24*). Protein load in gel slots was 4 μg (**Figure 1A**,**B**) or 8 μg (**Figure 1C**), and gels were stained with Coomassie brilliant blue R. Relative molecular mass (M_r) was estimated by comparison with molecular weight markers. Broad range markers included myosin (200 kDa), *β*-galactosidase (116.2 kDa), phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), and lysozyme (14.4 kDa). In all experiments, untreated BSA was used as control.

Tryptophan Fluorescence Spectra. Protein intrinsic fluorescence was performed by tryptophan excitation at 295 nm, and emission spectra were collected from 300 to 400 nm with 5 nm excitation and emission slits, using a Perkin-Elmer LS-50B fluorescence spectrophotometer (Waltham, MA). Each sample was dissolved in 50 mM PBS pH 7.2 and adjusted to 0.05 absorbance units to 280 nm. Emission spectra obtained with PBS were subtracted from those containing the treatments. All assays were done in triplicate (*25*).

Mass Spectrometry. Spectrometric analysis for untreated BSA and glycated BSA (BSA-Lac under dry-heat conditions for 28 days and BSA-Lac under wet-heat conditions for 20 h) was sent to the Arizona Proteomics Consortium (Proteomic Services, University of Arizona, USA). Matrix-assisted laser desorption-ionization time-of-flight (MALDI-TOF) mass spectra were acquired using an Applied Biosystems Voyager DE-STR (Framingham, MA), operating a 337 nm nitrogen laser. The dry sample pellets were resuspended in a solution of H_2O containing 0.1% trifluoroacetic acid (TFA) to give a final concentration of 2 μg/μL. The sample was then vortexed until the pellet dissolved completely. A 5 μL sample aliquot was mixed with an equal volume of a saturated α-cyano-4-hydroxycinnamic acid solution in 50% acetonitrile/50% water containing 0.1% TFA, and then 1 μL was spotted on the target plate and allowed to air-dry prior to mass analysis. Mass spectra were collected in linear mode

with an accelerating voltage of 25 000 V. The grid voltage was set at 90% with an extraction delay time of 800 ns. A minimum of 300 laser shots at 20 Hz was combined per mass spectra recorded.

Determination of Free Amino Groups. The amount of free amino groups present in nonglycated and glycated BSA was determined by the *ortho*-phthaldialdehyde (OPA) method (*26*). OPA reagent was prepared by mixing 25 mL of 0.1 M sodium borate, 2.5 mL of 20% SDS, 100 μL of 2-mercaptoethanol, and 40 mg of OPA (dissolved in 1 mL of methanol) and adjusting the final volume to 50 mL with distilled water. The OPA reagent was prepared fresh before use. Samples were adjusted to 0.2 absorbance units at 280 nm, and 100 μL aliquot was added to 1 mL of OPA reagent, incubated for 2 min at room temperature, and absorbance read at 340 nm. Blanks contained only the OPA reagent. Unreacted amino groups were estimated from a calibration curve done using different concentrations of free glycine. Results are average and standard deviation of three determinations.

Lectin Binding Assays. Recognition of BSA-Lac (BSA-glucose-β1−4-galactose) treatments by RCAI was achived by enzyme-linked lectinosorbent assay (ELLA) as described (*12*) with the following modifications. Fifty nanograms of each treatment was dissolved in 100 μL of 50 mM carbonate buffer pH 9.6 and used to coat microtiter plates. Plates were maintained overnight at 4 °C, washed four times with T-TBS (Tween 20 0.05%/Tris-HCl buffer saline 50 mM pH 7.5) and blocked using 300 μL of 1% BSA in T-TBS for 1 h at 37 °C. Plates were washed four times, incubated for 2 h at 37 °C with 100 μL (5 μg/mL) of biotinylated RCAI diluted in TBS, and washed again prior to the addition of 100 μL streptavidin peroxidase at a 1:2000 dilution in TBS for 1 h at 37 °C. Finally, plates were washed four times, and the color reaction developed using 100 μL of Sigma FAST OPD (one *o*-phenylenediamine tablet and one urea hydrogen peroxide/buffer tablet dissolved in 20 mL of water). Absorbance was read at 450 nm using a BIORAD ELISA plate reader. Commercial neoglycoprotein BSA-Galα(1−3)Gal was used as positive control. Results are average and standard deviation of two analyses.

***E. coli* K88 Adhesin Binding Assays.** Recognition of BSA-Lac treatments by *E. coli* K88 adhesins was accomplished by immobilizing bacteria solution on microtiter plates and assessing their ability to recognize biotinylated BSA-Lac treatments. Biotinylation of glycoconjugates was according to Hofmann et al. (*27*) by adding 4 mg of biotinamidocaproate *n*-hydroxysuccinimide ester (previously dissolved in 350 μL of dimethylsulfoxide) to 5 mg of glycated protein dissolved in 3 mL of PBS (50 mM phosphate buffer, 0.15 M NaCl, pH 7.2). After 3 h incubation at room temperature, treatments were dialyzed against PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Samples were frozen and stored at −40 °C until analysis. *E. coli* K88 strain, kindly donated by Dr. Carlos Eslava from Universidad Nacional Autonoma de Mexico, was maintained as frozen stocks. For the assays, bacteria were cultivated in tripticase soy agar and harvested by centrifugation after 24 h of incubation at 37 °C. Cells were suspended in PBS.

Bacteria were fixed to the polystyrene plates using glutaraldehyde (*28*). Preliminary experiments were conducted to determine the appropriate ratio of bacteria to glutaraldehyde. For these assays, bacteria solutions were adjusted from 0.2 to 0.7 absorbance units at 660 nm and glutaraldehyde solution from 0.2 to 1.2%. The best conditions were obtained with bacteria adjusted to 0.5 absorbance units at 660 nm (2×10^8 CFU/mL) and 1% glutaraldehyde; 100 μL of this solution was immobilized on microtiter plates and incubated for 1 h at room temperature. Plates were washed four times with T-PBS (0.05% Tween 20, 50 mM phosphate buffer, 0.15 M NaCl, pH 7.2) and blocked overnight at 4 °C using 300 μL of 1.5% BSA in T-PBS. Plates were incubated for 1.5 h at room temperature with 100 μL containing 1 μg of biotinylated protein, washed four times using T-PBS, and incubated again with 100 μL of streptavidin peroxidase at a 1:2000 dilution in PBS for 1 h at room temperature. Finally, the color reaction was developed as described before. Glycoproteins from pig duodenal mucin were used as positive control. Results are average and standard deviation of two analyses.

RESULTS AND DISCUSSION

Electrophoresis. Temperature and pH play crucial roles in the Maillard reaction (*29*, *30*). In general, an increase in temperature and pH leads to an increase of sugar and amino groups' reactivity.

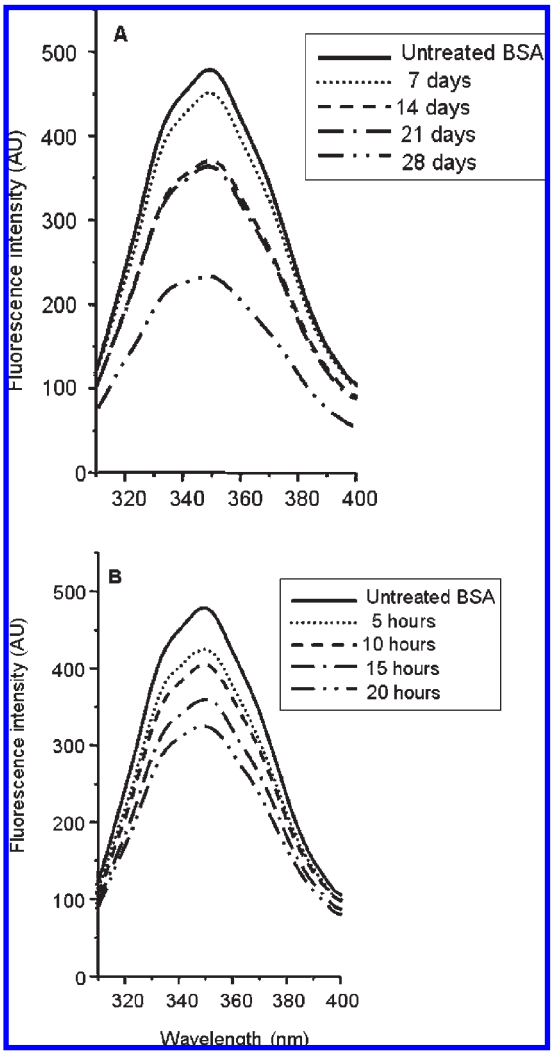

Figure 2. Intrinsic tryptophan fluorescence spectra of untreated and glycated BSA. The excitation wavelength was 295 nm, and emission was scanned from 300 to 400 nm. BSA samples were incubated with lactose at (**A**) 60 °C for 7, 14, 21, and 28 days or (**B**) under 43% relative humidity and 50 °C for 5, 10, 15, and 20 h.

Table 1. Mass Values for the Most Abundant Ions in its Monomer Form Obtained from Untreated BSA and BSA-Lac Products under Dry-Heat and Wet-Heat Conditions at the End of the Experiment

treatment	molecular mass (Da)	carbohydrate added[a] (molecules)
untreated BSA	66431.3	
BSA-Lac 28 days in dry-heat conditions	70797.1	13.47
BSA-Lac 20 h in wet-heat conditions	71049.1	14.25

[a] To determine the number of carbohydrate molecules added, mass difference (BSA-Lac − untreated BSA) was divided by 324.

At high pH, the carbohydrate open chain structure and the unprotonated amino groups are the reactive forms (*23*, *31*). Additionally, water activity also favors the reaction rate, allowing for greater mobility of reactants (*32*). In these studies, several conditions were tested to generate BSA-Lac products through the Maillard reaction.

As part of the characterization, SDS−PAGE was applied. Important changes in migration patterns of glycated BSA (BSA-Lac) were observed in SDS−PAGE (**Figure 1**). In general,

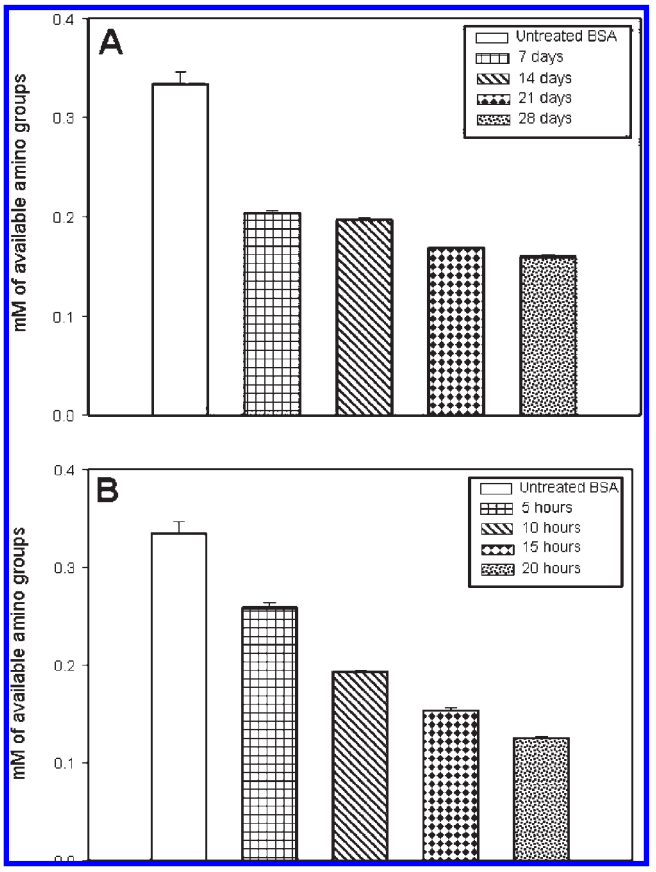

Figure 3. Available amino groups of untreated and BSA glycated with lactose at (**A**) 60 °C for 7, 14, 21, and 28 days or (**B**) under 43% relative humidity and 50 °C for 5, 10, 15, and 20 h.

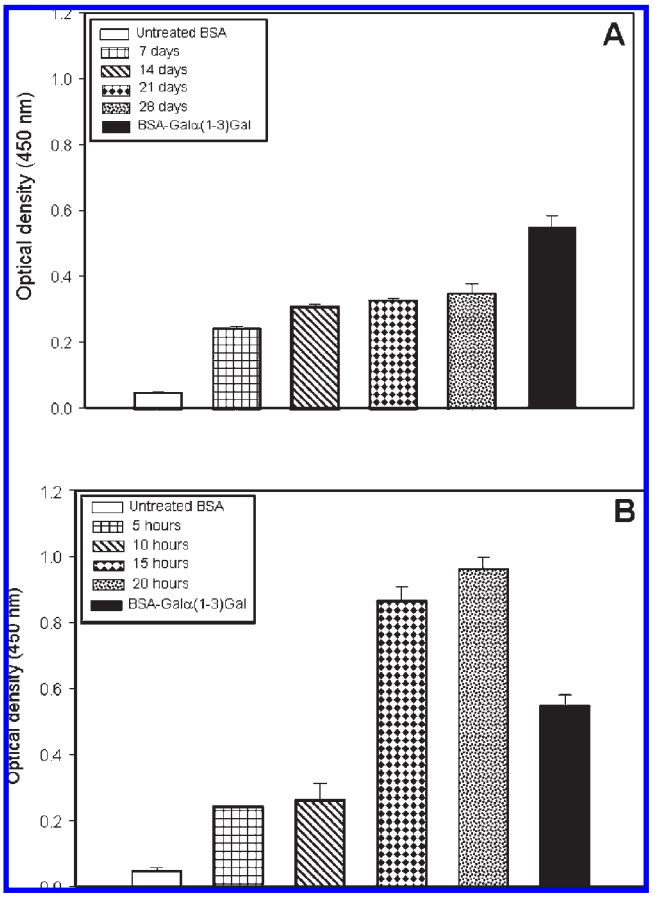

Figure 4. Recognition of BSA-Lac conjugates (50 ng) by *Ricinus communis* I lectin. BSA was glycated with lactose at (**A**) 60 °C for 7, 14, 21, and 28 days or (**B**) under 43% relative humidity and 50 °C for 5, 10, 15, and 20 h. Neoglycoconjugate BSA-Galα(1−3)Gal was used as positive control.

bands of glycated BSA were broader and more retarded than untreated BSA. Although the same amount of glycated protein was loaded, differences in band intensities for BSA-Lac monomers were observed (**Figure 1A**,**B**). At the end of the experimental conditions, the monomer mass was estimated to be 71.5 and 74.6 kDa for the dry-heating and wet-heating strategies, respectively (**Figure 1A**,**B**, lane 5). BSA-Lac produced under wet-heat conditions showed greater glycation heterogeneity (band broadening) and aggregation or polymer formation (as seen near and at the top of the gel). Protein polymerization was observed at extended heating times and when higher concentrations (8 μg) of protein were loaded to the gel (**Figure 1C**); this behavior is consistent with dimer and trimer formation probably caused by intermolecular covalent linkage formation. Sun et al. (*33*, *34*) studying ovalbumin glycation using wet-heat conditions observed a large amount of high molecular weight aggregates only in glycated samples, and they indicated that protein polymerization was due to incubating conditions and to formation of covalent bonds (i.e., sugar-lysine amino carbonyl and intermolecular SS bonds). Similar results have been reported for lactoglobulin glycation (*35*, *36*).

Tryptophan Fluorescence Spectra. Intrinsic tryptophan fluorescence emission spectra of glycated BSA with lactose under the used conditions are presented in **Figure 2**. Both conditions showed a time-dependent reduction in fluorescence intensity (FI). Similar results were reported by Sarabia-Sainz et al. (*12*) for conjugates of porcine serum albumin and lactose incubated under 43% RH and 60 °C for 0, 4, 6, and 8 h. Greater FI reduction was observed for dry-heating condition products, which could be attributed to partial loss of the protein native packing and shielding effect of the bound carbohydrate. This effect could also be due to the prolonged exposure to 60 °C. It is important to indicate that protein denaturation was not observed because none of the BSA-Lac treatments showed blue or red shifts in wavelength of maximum emission. Intrinsic fluorescence of most proteins is dominated by the surrounding environment of their tryptophan residues (indole nucleus), which are highly sensitive to solvent polarity, thus, the emission spectra of tryptophan residues are importants tools to study protein structure and dynamics (*37*).

Mass Spectrometry. The analysis of data obtained by MALDI-TOF included only the BSA-Lac molecular mass of the most abundant ion in its monomer form (**Table 1**). BSA-Lac glycated for 20 h under wet-heat conditions presented greater increase in mass (71 049.1 Da) than BSA-Lac glycated for 28 days under dry-heat conditions (70 797.1 Da). Lactose condensation leads to a mass increase of 324 Da, and the number of carbohydrate molecules added was estimated by comparing the mass difference of glycated and untreated protein. BSA-Lac conjugates showed 13.47 and 14.25 molecules of carbohydrate added for dry-heat and wet-heat conditions, respectively. Although differences were not significant (1 lactose), it is important to note that under the wet-heat conditions the conjugation was carried out in 20 h of incubation, while the dry-heat conditions required 28 days. These results are consistent with those obtained from SDS−PAGE of monomers while the polymer (dimers and trimers) mass remains to be evaluated.

Free Amino Groups. After protein glycation, the nonmodified amino groups could react with *ortho*-phenylendiamine (OPA reagent),

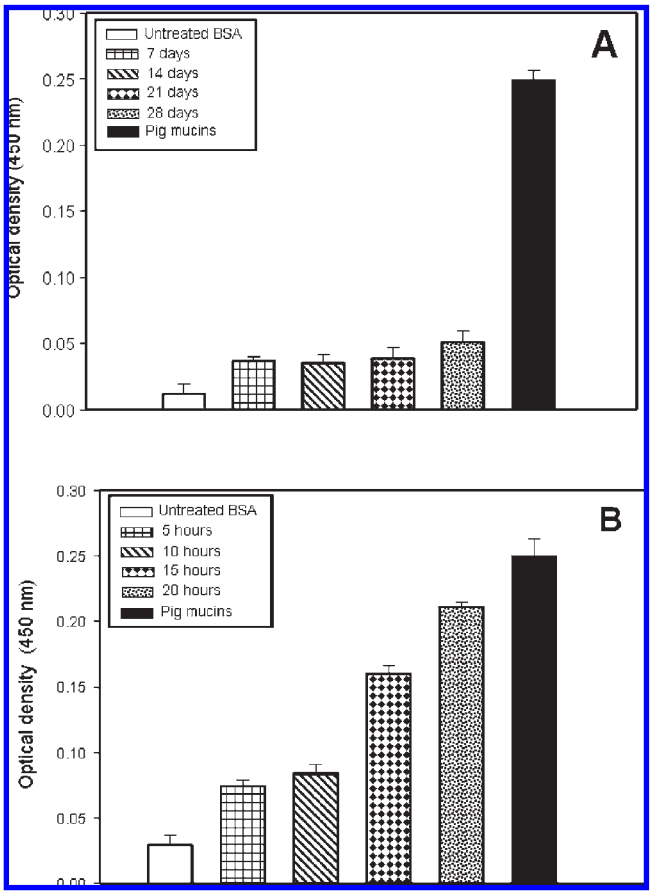

Figure 5. Biorecognition of BSA-Lac conjugates (1 μg) by *E. coli* K88 adhesins. BSA was glycated with lactose at (**A**) 60 °C for 7, 14, 21, and 28 days or (**B**) under 43% relative humidity and 50 °C for 5, 10, 15, and 20 h. Pig duodenal mucin was used as positive control.

producing compounds that have a maximum absorption at 340 nm (*38*). Thus, OPA was used to assess global glycation (monomers and polymers) for the different treatments. BSA glycated under dry conditions showed a significant decrease of free amino groups after the initial 7 days of incubation and remained similarly until the end of the experiment (**Figure 3A**). However, BSA glycated under wet conditions showed consistent and more pronounced reduction of available amino groups throughout, which indicates that a greater conjugation of these groups with lactose was achieved (**Figure 3B**). Therefore, glycation under wet conditions (higher water activity and pH) appeared more efficient. Similarly, reduction in the content of available amino groups during glycation of different proteins such as ovalbumin (*33*), β-lactoglobulin (*35*), and α-lactalbumin (*39*) has been reported.

Lectin Binding Assays. In order to evaluate if BSA-Lac products could be used for biological recognition, lectin binding assays were performed with RCAI (a galactose binding lectin). BSA-Lac from both conditions was recognized by RCAI, and greater interaction was observed with longer glycation time (**Figure 4**). Furthermore, wet-heat condition products showed more affinity for RCAI lectin than BSA-Lac produced under dry-heating conditions and a commercial glycoprotein BSA-Galα-(1−3)Gal used as positive control (this protein contains at least 14 carbohydrate epitopes bound). The greater interaction of BSA-Lac treatments could be explained by a larger number of lactoses available for interaction and also by the greater affinity of RCAI for β-galactose than to α-galactose (*40*).

Even though BSA-Lac monomers from both treatments contain similar number of lactoses added, the greater lectin interaction with those products obtained under wet conditions suggests that these lactoses are either more accessible and/or protein polymers are glycated and recognized by RCAI. The latter is consistent with OPA results.

***E. coli* K88 Adhesin Binding Assays.** K88 fimbrial adhesins are surface filaments with lectin activity that recognize specific glycoconjugates (glycoproteins or glycolipids) on the surface of intestinal cells. Adhesins allow bacteria to attach to carbohydrate receptors, colonize, and subsequently cause infection. All BSA-Lac products were recognized by *E. coli* K88 adhesins (**Figure 5**), as the lactose bound to proteins through the Maillard reaction leaves galactose available for biorecognition. Once more, BSA-Lac obtained under wet-heat conditions displayed greater interaction with *E. coli* K88 adhesins than those products obtained under dry-heat conditions. Also, the products obtained after 20 h of incubation showed similar interaction as pig mucins, *E. coli* K88 natural host (**Figure 5B**).

Grange et al. (*41*) reported that *E. coli* K88 adhesins bind preferentially to glycosphingolipids containing a linked *N*-acetylhexosamine, either *N*-acetylglucosamine, *N*-acetylgalactosamine, or galactose in the terminal position, thus specific carbohydrates that could compete for adhesion attachment site can be a useful alternative for disease prevention (*42*).

In this study, dry-heat and wet-heat conditions were evaluated for production of BSA-Lac conjugates through the Maillard reaction. The protein−carbohydrate conjugation appeared time-dependent, and glycoconjugates obtained from both conditions showed biorecognition by a soluble plant lectin (RCAI) and a bacteria-attached adhesin. However, BSA-Lac formed under wet conditions (50 °C and 43% RH) was promptly synthesized and more effectively recognized by the biological systems used. The strategy used in this work represents a simple method to obtain glycoconjugates for important applications involving carbohydrate recognition.

ABBREVIATIATIONS

BSA, bovine serum albumin; Lac, D-lactose; BSA-Lac, bovine serum albumin−lactose conjugate; RH, relative humidity; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; FI, fluorescence intensity; RCAI, *Ricinus communis* agglutinin I; ETEC, enterotoxigenic *Escherichia coli*; OPD, *o*-phenylenediamine dihydrochloride; OPA, *ortho*-phthaldialdehyde. MALDI-TOF, matrix-assisted laser desorption-ionization time-of-flight; TFA, trifluoroacetic acid; ELLA, enzyme-linked lectinosorbent assay; T-TBS, Tween 20 0.05%/Tris-HCl buffer saline 50 mM, pH 7.5; T-PBS, Tween 20 0.05%, 50 mM phosphate buffer, 0.15 M NaCl, pH 7.2.

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