DOI:10.1021/jf102869f

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Conjugates of Bovine Serum Albumin with Chitin Oligosaccharides Prepared through the Maillard Reaction

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Chitin neoglycoconjugates (BSA−CO) were obtained by the conjugation of bovine serum albumin (BSA) with chitin oligosaccharides (CO) through the Maillard reaction (nonenzymatic glycation). CO produced by acid hydrolisis of chitin were fractionated using an ultrafiltration membrane system (1−3 kDa cutoff). The Maillard reaction was carried out by heating a freeze-dried mixture containing BSA and CO at 60 °C (under 43% relative humidity for 6 and 12 h). BSA−CO were characterized by available amino groups content, intrinsic tryptophan emission spectra, gel electrophoresis, and mass spectrometry. Biological assays included interaction with wheat germ agglutinin (WGA) and with bacterial adhesins of *Escherichia coli* K88$^+$ and *Salmonella choleraesuis*. Glycation of BSA was revealed by reduction of available amino groups and fluorescence intensity and also retarded migration through SDS-PAGE. Conjugation of BSA with chitin oligomers appeared to be time dependent and was confirmed by mass spectrometry, by which molecular mass increase for monomers and dimers was observed. Monomers were estimated to contain either one or two glycation sites (at 6 and 12 h of treatment, respectively), with one or two tetrasaccharide units attached. Consequently, dimers showed two or four glycation sites. BSA−CO presented biological recognition by WGA and *E. coli* K88$^+$ and *S. cholerasuis* adhesins. The strategy used in this work represents a simple method to obtain glycoconjugates to study applications involving protein−carbohydrate recognition.

KEYWORDS: Chitin oligosaccharides; glycated bovine serum albumin; biorecognition

INTRODUCTION

Chitin is a polysaccharide synthesized from units of *N*-acetylglucosamine (GlcNAc) in β1−4 linkages. This homopolymer is widely distributed in cell walls of fungi and exoskeletons of crustaceans and invertebrates (*1*). Chitosan, a copolymer of GlcNAc (~20%) and glucosamine (GlcN, 80%) residues, is derived from deacetylation of chitin with enzyme (*2*) or alkaline solution treatment (*3*).

The nontoxic, biodegradable and biocompatible properties of chitin and chitosan provide much potential for many food, pharmaceutical, and biotechnology applications (*4*). Despite its abundance, utilization of chitosan is restricted, owing to its high molecular weight, high viscosity, and, thus, low absorption for in vivo applications (*5*). Studies on chitin and chitosan have attracted interest for converting them to oligosaccharides, because oligosaccharides are not only water-soluble but also possess versatile properties such as antitumor activity (*6*), immunoenhancing effects (*7*), and improvement of protective effects against infection with some pathogens (*8*).

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Chitin oligosaccharides (CO) such as hexa-*N*-acetylchitohexaose, $(GlcNAc)_6$, has immunopotentiating and antitumor functions (*9*). *N*-Acetylchito-oligosaccharides and chito-oligosaccharides inhibit the growth of fungi and phytopathogens (*10*) and elicit defense mechanisms in plants by activating the production of pisatin, chitinase, and phytoalexin (*11*). Oligosaccharides also affect the mitogenic response and chemotactic activities of animal cells (*12*).

In terms of antibacterial activity, Kumar et al. (*5*) reported that a chito-oligomeric−monomeric mixture showed growth inhibitory activity toward *Bacillus cereus* and *Escherichia coli*, that is to say, they were effective against Gram-positive and Gram-negative bacteria, and optimum growth inhibition was observed with chito-oligomers of higher polymerization degrees.

Chito-oligosaccharides have also been used to synthesize glycolipids and to form carbohydrate−protein conjugates with various biological activities (*13*, *14*). Gorbach et al. (*15*) synthesized neoglycolipids containing chito-oligosaccharides that presented immunostimulating and antitumor activities, arguing that fatty acid residues may intensify or modify the biological function of oligosaccharides.

Recently, proteins such as serum albumin (porcine and bovine) chemically modified with disaccharides were biorecognized by

 Published on Web 11/02/2010 © 2010 American Chemical Society

plant lectins and bacterial adhesins (*16*−*18*). Furthermore, porcine serum albumin conjugated with lactose could compete in vitro with intestinal bacterial natural ligands (*16*).

In this study, neoglycoconjugates were prepared using bovine serum albumin (BSA) and *N*-acetylchitin oligosaccharides through the Maillard reaction to determine if they could be recognized by *N*-acetylglucosamine-specific lectins and bacterial adhesins in an attempt to be used as competing agents for microorganism attachment.

MATERIALS AND METHODS

Materials. All reagents used were of analytical grade. BSA, fetuin, and chitin (from crab shells) were purchased from Sigma-Aldrich (St. Louis, MO). Broad range markers were from Bio-Rad (Hercules, CA), and biotin -labeled wheat germ agglutinin (WGA) was acquired from Vector (Burlingame, CA). α-Amylase (EC 3.2.1.1, 1,4-α-D-glucan glucanohydrolases) from a strain of *Bacillus amyloliquefaciens* was from Roche Diagnostics (Basel, Switzerland). *E. coli* K88$^+$ was kindly donated by Dr. Carlos Eslava from Universidad Nacional Autonoma de Mexico, and *Salmonella choleraesuis* was isolated from pigs on farms in the state of Sonora; both strains were kept as frozen stocks.

Preparation of CO. Chitin was treated with acid according to the method of Scheel and Theim (*19*). Chitin (2 g) was ground to a fine powder, placed into a flask, dissolved in 16 mL of 6 M HCl at 30 °C, and stirred for 10 min. This solution was further incubated for 110 min at 40 °C under continuous stirring. Prior to neutralization, the reaction mixture was placed in a water bath containing an ice/salt mixture for a few minutes, and then a 50% aqueous NaOH solution was carefully added to the continuously stirred reaction mixture. Insoluble material was removed from the chitin hydrolysate by centrifugation at 10000*g* for 25 min at 5 °C.

The supernatant was applied to a tandem ultrafiltration system, which membranes had cutoffs of 3 and 1 kDa, respectively. CO obtained were those that passed through the 3 kDa membrane but were retained by the 1 kDa membrane (*20*). Hydrolysates were desalted by ultrafiltration using a membrane of 1 kDa cutoff and then subjected to freeze-drying.

Fluorophore-Assisted Carbohydrate Electrophoresis (FACE). Freeze-dried chitin oligosaccharides (500 μg) were labeled by adding 5 μL of a stock solution containing 0.15 M 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt (ANTS) in acetic acid/water (3:17, v/v) and 5 μL of freshly made 1.0 M sodium cyanoborohydride in dimethyl sulfoxide (DMSO) followed by incubation at 37 °C for 16 h according to the method of Jackson (*21*). The reaction mixture was dried under vacuum for 45 min in a speed-vac at 40 °C and dissolved in 8 μL of glycerol/water (1:4, v/v). ANTS-labeled oligosaccharides (8 μL) were loaded on a 35% polyacrylamide gel without SDS (0.5 mm thick), according to the method of Laemmli (*22*). Gel electrophoresis was conducted at 4 °C for 6 h at 20 mA. Gel was visualized using a Kodak transilluminator UV/white 2020D. To identify the oligosaccharide size, a standard ladder of glucose oligomers was prepared by enzyme hydrolysis of starch with α-amylase as described by Jackson (*21*); furthermore, 200 pmol maltopentaose (M5) and maltohexaose (M6) standards were used.

Synthesis of BSA−CO. BSA and CO mixtures (1:2 w/w) dissolved in 20 mM Na_2CO_3 buffer (pH 9.0) were freeze-dried and later incubated at 60 °C for 6 and 12 h under 43% relative humidity. Humidity conditions were controlled using a desiccator containing a saturated K_2CO_3 solution. After incubation, samples were dissolved in 5 mL of water, extensively dialyzed using a membrane of 10 kDa cutoff (Sigma) to remove salts and unbound oligosaccharides, and stored at −40 °C until analysis. Experiments were done in duplicate.

Electrophoresis of BSA−CO. SDS-PAGE in 8% polyacrylamide gel was performed under reducing conditions (*22*). Protein load in gel slots was 4 μg, and gel was stained with Coomassie brilliant blue R. Broad range molecular weight markers included myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase *b* (97.4 kDa), BSA (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 included as control.

Determination of Available Amino Groups. The amount of free amino groups in nonglycated and glycated BSA was determined by using the *o*-phthaldialdehyde (OPA) method (*23*). OPA reagent was prepared by mixing 25 mL of 0.1 M sodium borate, 2.5 mL of 20% SDS, and 100 μL of 2-mercaptoethanol with 40 mg of OPA (dissolved in 1 mL of methanol). The final volume was adjusted to 50 mL in distilled water. The OPA reagent was prepared fresh before use. Samples were adjusted to 0.2 absorbance unit at 280 nm, and 100 μL aliquots were added to 1 mL of OPA reagent and incubated for 2 min at room temperature; absorbance was read at 340 nm. Blanks contained only the OPA reagent. Unreacted amino groups were estimated from a calibration curve done using different concentrations of glycine. Results were the average and standard deviation of three determinations.

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 PBS (50 mM phosphate buffer, 0.15 M NaCl, pH 7.2) and adjusted to 0.05 absorbance unit to 280 nm. Emission spectra collected with PBS was subtracted from those containing the treatments (*24*). All assays were done in triplicate.

Mass Spectrometry. For spectrometry analysis of BSA−CO, samples were sent to the Arizona Proteomics Consortium (Proteomic Services, University of Arizona). 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 containing 0.1% trifluoroacetic acid (TFA) to give a final concentration of 2 μg/μL. The sample was 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 25000 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 were combined per mass spectra recorded.

Lectin Binding Assays. Recognition of BSA−CO conjugates by WGA was achived by enzyme-linked lectinosorbent assay (ELLA) as described by Sarabia-Sainz et al. (*16*). Briefly, 500 ng of each treatment was dissolved in 100 μL of 50 mM Na_2CO_3 buffer, pH 9.6, and used to coat microtiter plates. Plates were kept overnight at 4 °C, washed four times with T-TBS (0.05% Tween 20 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 with T-TBS, incubated for 2 h at 37 °C with 100 μL (5 μg/mL) of biotinylated WGA diluted in TBS (Tris-HCl buffer 50 mM, pH 7.5), and rinsed again with T-TBS, prior to the addition of 100 μL of streptavidin peroxidase (1:2000 in TBS) for 1 h at 37 °C. Finally, plates were washed four times, and the color reaction was 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 Bio-Rad ELISA microplate reader model 680 (Hercules, CA). Fetuin glycoprotein was used as positive control. Results are an average of two analyses.

***E. coli* K88$^+$ and *S. choleraesuis* Adhesin Binding Assays.** Recognition of BSA−CO by *E. coli* K88$^+$ and *S. choleraesuis* adhesins was accomplished by bacteria immobilization on microtiter plates and assessing their ability to recognize biotinylated BSA−CO. Biotinylation of glycoconjugates was performed according to the method of Hofmann et al. (*25*) by adding 4 mg of biotinamidocaproate *n*-hydroxysuccinimide ester (previously dissolved in 350 μL of DMSO) to 5 mg of glycated protein dissolved in 3 mL of PBS. After 3 h of incubation at room temperature, treatments were dialyzed in PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Conjugates were frozen and stored at −40 °C until analysis.

For assays, bacteria were grown in tripticase soy agar and harvested by centrifugation after 24 h of incubation at 37 °C. Cells were suspended in PBS and adjusted to 0.5 absorbance unit at 660 nm (2×10^8 CFU/mL); 100 μL ot this solution was immobilized on microtiter plates using 1% glutaraldehyde and incubated for 1 h at room temperature as reported by Ledesma-Osuna et al. (*18*). 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. Plates were washed four times using T-PBS and incubated with 100 μL of streptavidin peroxidase 1:2000 dilution for 1 h at

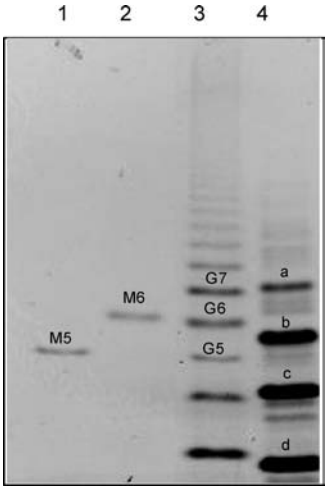

Figure 1. FACE profiles of ANTS-labeled carbohydrates. Lanes: 1, maltopentaose (M5); 2, maltohexaose (M6); 3, standard ladder of glucose oligomers; 4, chitin-oligosaccharides obtained by acid hydrolysis of chitin.

room temperature. Finally, the color reaction was developed as described before. Glycoproteins isolated from pig duodenal mucin according to the method of Sarabia-Sainz et al. (*16*) were used as positive control. Results are an average of two analyses.

RESULTS AND DISCUSSION

FACE Analysis. FACE of ANTS-labeled glycans is widely used for the analysis of oligosaccharides isolated from different sources. Identification of structures is based on their electrophoretic mobility in comparison with reference standards. To analyze CO obtained from acid hydrolysis of chitin, these were labeled with ANTS and then separated by electrophoresis in a polyacrylamide gel and detected by fluorescence under UV illumination (**Figure 1**). Standards of M5 (lane 1), M6 (lane 2), and a ladder of glucose oligomers (lane 3) were included in the experiment to provide a convenient reference marker of the electrophoretic separation. The FACE profile of chitin oligosaccharides showed four major bands (lane 4) assigned as oligosaccharides a, b, c, and d. CO size was estimated by comparison with standards in units of degree of polymerization (DP). Thus, M5 (five glucose units) has a DP of 5, and M6 (six glucose units) has a DP of 6. In this way, the oligosaccharides obtained by chitin hydrolysis ranged from 4 to 7 DP.

These results were consistent with the findings of Ngo et al. (*26*) obtained under similar conditions. Their main hydrolysis products were in a range from tetramer to heptamer corresponding to masses between 853.63 (for DP = 4) and 1462.52 Da (for DP = 7) estimated by MALDI-TOF.

Determination of Available Amino Groups. During glycation, side amino acid groups of proteins react with carbonyl groups of sugars to form a conjugate (*27*). The remaining amino group content can be estimated by reaction with the OPA reagent to evaluate global glycation. The assay is based on the formation of 1-alkylthio-2-alkylisoindoles generated by the reaction of amino groups with OPA in the presence of a thiol component; the products possess a maximum absorption at 340 nm (*23*). BSA glycated with CO showed a reduced content of available amino groups relative to untreated BSA (**Figure 2A**). The longer the heating time (12 h), the fewer available amino groups were detected, which indicates a higher glycation. Similar results were obtained during glycation of BSA with different carbohydrates (*17*, *28*).

Tryptophan Fluorescence Spectra. Fluorometric studies were performed to characterize changes in tryptophan surroundings

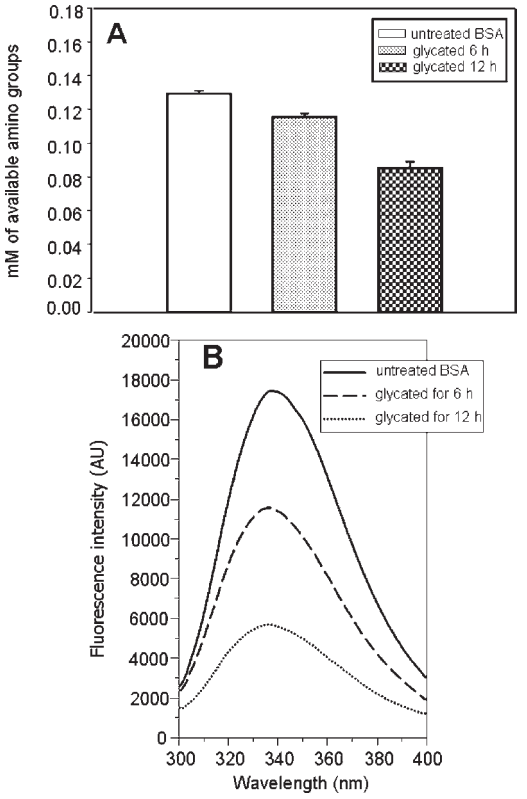

Figure 2. Changes in availability of amino groups (**A**) and intrinsic tryptophan fluorescence (**B**) of BSA−CO treatments. BSA was glycated with CO under 43% of relative humidity and 60 °C for 6 and 12 h.

with increased glycation. The fluorescence emission espectra of conjugates are shown in **Figure 2B**. Emission fluorescence scans showed a reduction in the maximum fluorescence intensity in glycation treatments. Reductions of about 35 and 70% of the initial fluorescence for BSA−CO 6 and 12 h treatments, respectively, were observed. Also, the maximum fluorescence peak presented a slight shift to the left (blue shift). A blue shift of the maximum emission is interpreted as the shielding of tryptophan residues from the aqueous phase by the protein (*29*). Thus, a reduction in fluorescence intensity suggests that the glycation affected partially the side chains of proteins in tertiary structure, without great disruption of native structure. Similar results were observed during glycation of ovalbumin, whey proteins, and human, porcine, and bovine serum albumins (*16*, *18*, *24*, *30*−*32*).

SDS-PAGE. Gel electrophoresis was used to evaluate the extent of glycation as well as visualize a potential protein aggregation resulting from the glycation treatment. Slight differences in migration patterns of glycated BSA with respect to untreated protein were observed in SDS-PAGE (**Figure 3A**). BSA−CO bands migrated broader and more retarded than untreated BSA, indicating that glycated samples contain a range of protein molecules with different numbers of coupled sugar residues. Even though all lanes were loaded with the same amount of total protein, the monomeric band (at 66.2 kDa) was more intense in the treatment for 12 h. Additionally, glycated samples showed protein bands with higher molecular mass resulting from aggregation or polymer formation at near the top of the gel. This behavior is consistent with dimer and trimer formation. Although SDS-PAGE showed no appreciable changes in migration of glycated monomers, aggregate formation (dimers and trimers) observed might also be glycated, which explains the higher glycation obtained by using the OPA method. The high molecular weight compounds observed in the SDS-PAGE could be attributed

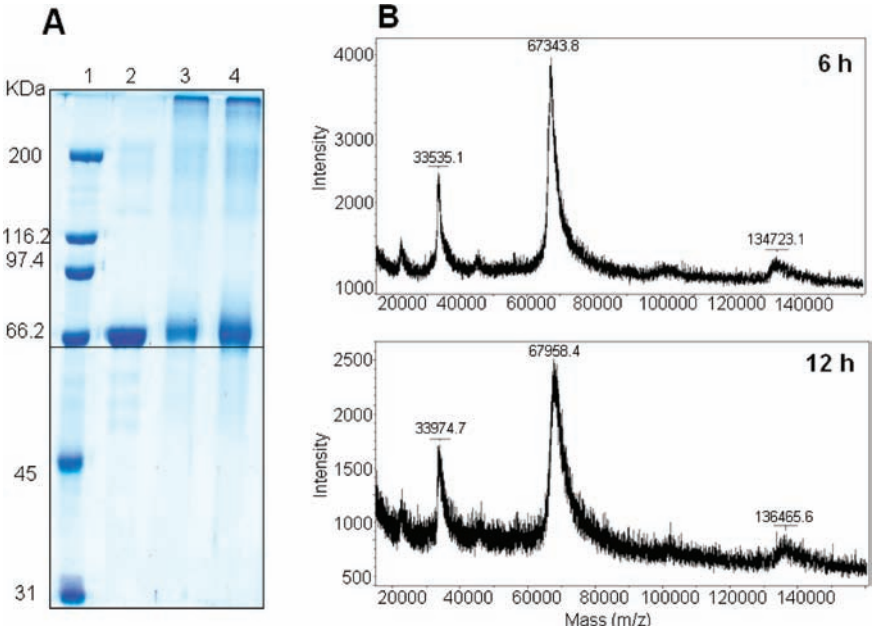

Figure 3. Characterization of BSA−CO. (**A**) SDS-PAGE in 8% gel. Lanes: 1, molecular weight markers; 2, untreated BSA; 3, 6 h glycated BSA; 4, 12 h glycated BSA. Protein loading was 4 μg. (**B**) MALDI-TOF spectra of BSA−CO conjugates. BSA was glycated with CO under 43% relative humidity and 60 °C.

Table 1. Mass Values for the Most Abundant Ions of Glycated BSA Monomers and Dimers and Estimation of Number of Tetrasaccharides (4GlcNAc) Attached

treatment	monomer (4GlcNAc)[a]	dimer (4GlcNAc)[a]
untreated BSA	66431.3 Da[b] (0)	132862.6 Da (0)
BSA−CO 6 h	67343.8 Da (1)	134723.1 Da (2)
BSA−CO 12 h	67958.4 Da (2)	136465.6 Da (4)

[a] For estimation of the number of tetrasaccharides (4GlcNAc) attached, the mass was 853.63 Da (*26*). [b] Da, mass in daltons.

to proteins covalently cross-linked through an amino−carbonyl reaction (*33*). Also, Sun et al. (*31*) reported that in the cross-linking of proteins, the formation of disulfide bonds could also be involved.

Mass Spectrometry. The MALDI-TOF analysis of conjugates is shown in **Figure 3B** and **Table 1**. **Figure 3B** depicts the spectra obtained from BSA glycated with chitin oligosaccharides under 43% relative humidity and 60 °C for 6 and 12 h. In the spectra, the masses for monomer and dimer forms of conjugates are observed. The spectrum of untreated BSA (data not shown) indicated a monomer molecular mass of 66431.3 Da. Thus, it was possible to estimate the increase in mass for monomers and dimers in BSA−CO. Taking into consideration that the oligosaccharide with 4 glucose units (DP = 4) is more reactive and could be attached to the protein, the number of sites that were modified was calculated (**Table 1**). The oligosaccharide with DP = 4 was chosen considering that the reactivity is greater at lower DP, to calculate the number of modified sites, it was considered a mass of 853 Da according to that reported by Ngo et al. (*26*) for the hydrolysis and separation of chitin under similar conditions. On the basis of these considerations, protein monomers contained one and two glycated sites at 6 and 12 h, respectively, and, consequently, two and four sites for protein dimers. It is important to note that the increment in the mass of the dimers confirmed that these were also glycated.

Lectin Binding Assays. To determine whether BSA−CO were recognized by specific lectins, an ELLA was conducted.

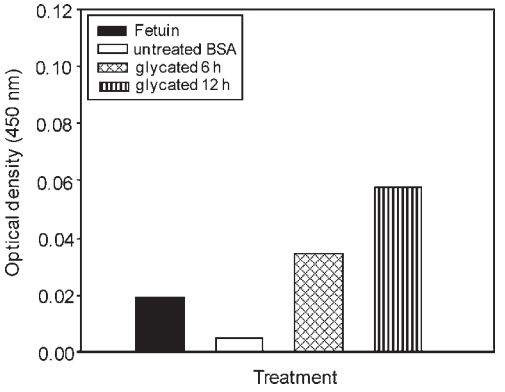

Figure 4. Recognition of BSA−CO by WGA. BSA was glycated with CO under 43% relative humidity and 60 °C for 6 and 12 h. Fetuin was used as positive control.

WGA has specificity toward chitobiose, chitotriose, and GlcNAc structures (*34*); thus, the obtained conjugates were recognized with greater affinity than fetuin, a glycoprotein used as positive control (**Figure 4**). Furthermore, a greater interaction with longer glycation time was observed. These results were consistent with data obtained using MALDI-TOF and available amino groups.

***E. coli* K88$^+$ and *S. choleraesuis* Adhesin Binding Assays.** Bacterial adhesion assays were performed to evaluate whether BSA−CO can be recognized by *E. coli* K88$^+$ and *S. choleraesuis* adhesins. It has been reported that oligosaccharides obtained from chitin hydrolysis have antimicrobial activities (*35*−*37*). Although the mechanism involved in these processes is not clear, the biological recognition by bacterial adhesins might be implicated. Fimbrial adhesins are filamentous surface appendages having lectin activity that allows attachment to specific glycoconjugates (ligands). Attachment is the initial step in the colonization and subsequent infection (*38*). Biorecognition assays showed that BSA−CO were recognized by *E. coli* K88$^+$ and

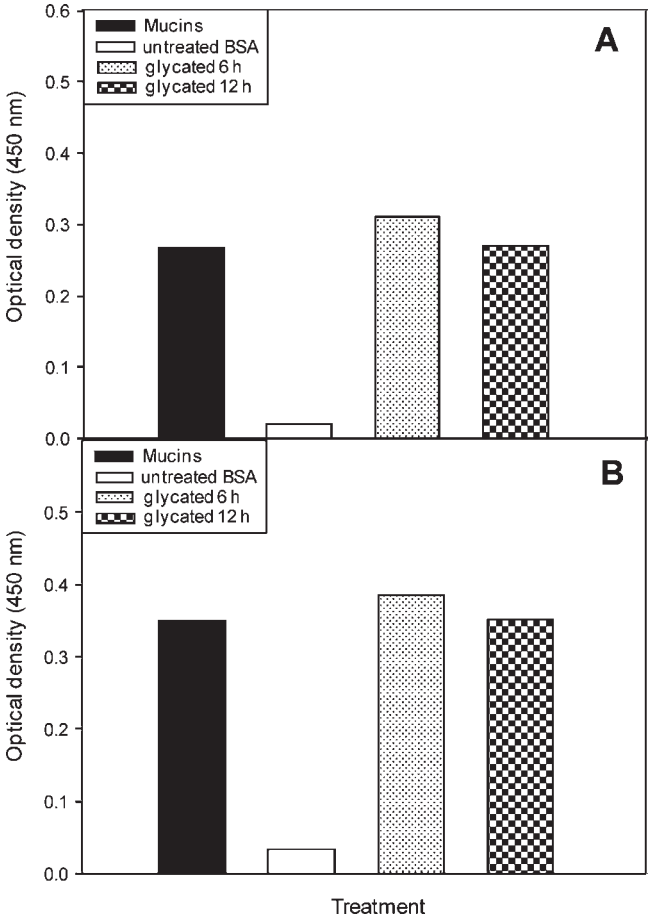

Figure 5. Recognition of BSA−CO conjugates by *E. coli* K88$^+$ (**A**) and *S. cholerasuis* (**B**) adhesins. Bacteria level was adjusted at 0.5 unit of absorbance, and 100 μL was adsorbed in ELISA plate wells and then overlaid with biotinylated BSA−CO treatments obtained under 43% relative humidity and 60 °C for 6 and 12 h. Glycoprotein from pig duodenal mucin was used as positive control.

S. choleraesuis adhesins (**Figure 5**). This interaction was similar to that of mucins, which are natural ligands for these microorganisms, and was not observed in untreated protein. Slight differences between BSA−CO treatments were observed. It has been reported that some enterotoxigenic *E. coli* expressing the F17-G adhesin (at the tip of flexible F17 fimbriae) mediates binding to *N*-acetyl-β-D-glucosamine-presenting ligands on the microvilli of the intestinal epithelium of ruminants (*39*). Also, Grange et al. (*40*) reported *E. coli* K88$^+$ adhesins preferentially bind to glycosphingolipids containing either *N*-acetyl-β-glucosamine or *N*-acetylgalactosamine at the terminal position. On the other hand, it has been reported that *Salmonella* can express multiple fimbriae; however, only the mannose specificity for type 1 fimbriae has been determined. Therefore, some other fimbriae could be involved in GlcNAc recognition (*41*).

This paper describes a simple way to generate conjugates of BSA with chitin oligosaccharides. Chitin oligosaccharides obtained by acid hydrolysis were 4−7 DP and actively participated in the Maillard reaction. Both BSA monomers and dimers were glycated. The protein−carbohydrate conjugation appeared to be time dependent, and neoglycoconjugates showed recognition by WGA, a plant lectin, and both *E. coli* K88$^+$ and *S. choleraesuis* adhesins. Neoglycoconjugates obtained could be used to study protein carbohydrate interactions as well as to search for prophylactic agents of bacterial infections in which GlcNAc recognition is involved.

ABBREVIATIONS USED

BSA, bovine serum albumin; CO, chitin oligosaccharides; BSA−CO, bovine serum albumin−chitin oligosacharides conjugates; WGA, wheat germ agglutinin; *E. coli* K88$^+$, *Escherichia coli* K88$^+$; *S. choleraesuis*, *Salmonella choleraesuis*; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; GlcNAc, *N*-acetylglucosamine; GlcN, glucosamine; M5, maltopentaose; M6, maltohexaose; OPD, *o*-phenylenediamine dihydrochloride; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid disodium salt; FACE, fluorophore-assisted carbohydrate electrophoresis; DMSO, dimethyl sulfoxide; OPA, *o*-phthaldialdehyde; DP, degree of polymerization; CFU, colony-forming unit; MALDI-TOF, matrix-assisted laser desorption ionization−time-of-flight; DE-STR, delayed extraction technology, TFA, trifluoroacetic acid; ELLA, enzyme-linked lectinosorbent assay; T-TBS, Tween 20 0.05%/Tris-HCl buffer 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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Received for review August 6, 2010. Revised manuscript received September 28, 2010. Accepted October 4, 2010. This work was supported financially by the National Council of Science and Technology of Mexico, CONACYT, under Project P47998-Q, which is also thanked for the scholarship for Ph.D. studies. Mass spectral analyses were performed by the Arizona Proteomics Consortium and supported by NIEHS Grant ES06694, NCI Grant CA023074, and the BIO5 Institute of the University of Arizona.