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Characterization of bovine serum albumin glycated with glucose, galactose and lactose

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The non-enzymatic reaction between reducing sugars and proteins, known as glycation, has received increased attention from nutritional and medical research. In addition, there is a large interest in obtaining glycoconjugates of pure well-characterized oligosaccharides for biological research. In this study, glycation of bovine serum albumin (BSA) by D-glucose, D-galactose and D-lactose under dry-heat at 60°C for 30, 60, 120, 180 or 240 min was assessed and the glycated products studied in order to establish their biological recognition by lectins. BSA glycation was monitored using gel electrophoresis, determination of available amino groups and lectin binding assays. The BSA molecular mass increase and glycation sites were investigated by mass spectrometry and through digestion with trypsin and chymotrypsin. Depending on time and type of sugar, differences in BSA conjugation were achieved. Modified BSA revealed reduction of amino groups' availability and slower migration through SDS/PAGE. D-Galactose was more reactive than D-glucose or D-lactose, leading to the coupling of 10, 3 and 1 sugar residues, respectively, after 120 minutes of reaction. BSA lysines (K) were the preferred modified amino acids; both K256 and K420 appeared the most available for conjugation. Only BSA-lactose showed biological recognition by specific lectins.

Keywords: glycation, Maillard reaction, bovine serum albumin glycoconjugates

INTRODUCTION

Glycoconjugates are the most functionally and structurally diverse molecules in nature. It is well established that protein- and lipid-bound saccharides play essential roles in many molecular processes that impact eukaryotic biology and some diseases (Varki, 1993). Also, in biological recognition processes, glycans are important in the binding of bacteria, toxins or viruses to mammalian cell surface glycans and in the specific recognition of a glycoprotein or glycolipid by cell surface receptors (Varki *et al*., 1999).

In glycoproteins, sugars are linked to defined amino acids through complex enzymatic reactions. N-Glycans are covalently linked to asparagine residues of a polypeptide chain within a consensus peptide sequence. O-Glycans are typically coupled to the hydroxyl group of serine, threonine or tyrosine residues (Taylor & Drickamer, 2006).

In contrast to glycosylation, protein glycation is the non-enzymatic reaction between amino groups of proteins and reducing sugars. Glycation is commonly recognized as the Maillard reaction (Finot, 2005). In this reaction, the carbonyl group of a sugar interacts with the nucleophilic amino group of the amino acid, producing N-substituted glycosylamine (Schiff base) which is labile and may undergo two sequential rearrangements, yielding a reasonably stable aminoketose — the Amadori product (Fayle & Gerrard, 2002). Certain protein groups are particularly prone to gly-

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Abbreviations: BSA, bovine serum albumin; OPA, *ortho*-phthaldialdehyde; SDS/PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; MALDI-TOF, matrix assisted laser desorption/ionization-time of flight; MS, mass spectrometry; PBS, phosphate-buffered saline.

cation; they include terminal amino groups and lysine side chains (Frister *et al.,* 1988). Arginine side chains can be glycated as well (Tagami *et al.,* 2000).

There is a large interest in obtaining glycoconjugates containing pure well-characterized oligosaccharides for use in biological studies, however, enzymatic synthesis is costly and complicated, since the number of glycosyltransferases available is limited (Ernst & Oehrlein, 1999). In contrast, there is evidence that under physiological conditions glucose reacts non-enzymatically with a wide variety of proteins to form glycated products.

In humans, serum proteins with slow turnover rates that are exposed to high concentrations of glucose are particularly susceptible to non-enzymatic glycation. In patients with diabetes mellitus, hemoglobin, human serum albumin, low density lipoproteins, lens crystallin and various forms of collagen are glycated. These protein modifications appear to contribute to the long-term complications of these patients (Shaklai *et al.,* 1984).

On the other hand, from the standpoint of food technology, non-enzymatic coupling of glucose and other carbohydrates to proteins has been reported to improve their functional properties, such as thermal stability, emulsifying and foaming properties, and water-holding capacity (Kato *et al.,* 1993; 1995; Wooster & Augustin, 2006). The Maillard reaction occurs frequently during industrial processing, prolonged storage or domestic cooking, sometimes also enhancing food properties through color, aroma and flavor (Finot, 2005).

Albumin, owing to its abundance in serum, is one of proteins undergoing glycation and conjugation at multiple sites (Iberg & Fluckiger, 1986). BSA glycation has been used to change its functional properties, especially foaming properties (Berthold *et al.,* 2007). Other proteins, like β-lactoglobulin, glycated at 60°C to accelerate the Maillard reaction, showed improvement of functional properties that were related to the sugar added (Chevalier *et al.,* 2001b; Chobert *et al.,* 2006).

Moreover, neoglycoconjugates from glycated BSA could be used as antigens for immunization, as components in diagnostic assays or anti-adhesion therapeutic drugs (Paschinger *et al.,* 2005; Sharon, 2006). Recently it has been reported that *Escherichia coli* K88 containing a specific lectin-like adhesin for β-galactose, recognize glycated porcine albumin (Sarabia-Sainz *et al.*, 2006). It is therefore of importance to investigate whether there are differences in recognition when mono- and disaccharides are attached to proteins. Non-enzymatic glycation could be a simple method to obtain glycoconjugates that provide well-defined materials for research of their biological properties.

MATERIALS AND METHODS

Materials. All reagents were analytical grade. D-Galactose (Gal) was from Fluka and Riedel-de-Haën. Bovine serum albumin (BSA), D-glucose (Glc) and D-lactose (Lac) were from Sigma Chemicals Co. (St Louis, MO, USA). Broad range markers were from BioRad (Hercules, CA, USA). Biotin-labeled *Ricinus communis* I, *Griffonia simplicifolia* I and *Lens culinaris* lectins were purchased from Vector (Burlingame, CA, USA).

Glycation. Glycation treatments were conducted according to Kanska & Boratyński (2002). Briefly, 150 μl of BSA (20 mg/ml) was mixed with 150 μl of sugar solution (40 mg/ml Glc or Gal, 80 mg/ml Lac), then 150 μl of 0.1 M phosphate buffer, pH 8.0, was added. Samples were frozen at –40°C, freeze-dried and heated at 60°C for 30, 60, 120, 180 or 240 min. After heating, samples were dissolved in 300 μl of water, dialyzed to remove salts and unbound sugar and kept frozen at –40°C until use. All experiments were done in duplicate, at least. Untreated BSA (without heating and carbohydrates) was used as control. The protein content of samples was determined by the dye binding method (Bradford, 1976), using bovine serum albumin as standard.

Determination of available amino groups. Free amino groups of glycated samples were determined by the *ortho*-phthaldialdehyde (OPA) method (Fayle *et al.,* 2001). OPA reagent was prepared fresh before use by mixing 25 ml of 0.1 M sodium borate, 2.5 ml of 20% SDS, 100 μl of 2-mercaptoethanol and 40 mg OPA (dissolved in 1 ml of methanol) and adjusting the final volume to 50 ml with distilled water. An aliquot of samples containing 50 μg of protein was adjusted to 1 ml with OPA reagent, incubated for 2 min at room temp. and absorbance read at 340 nm against a blank containing the OPA reagent. Untreated BSA (control) was assumed to have 100% available amino groups.

Gel electrophoresis. Glycoconjugates from each treatment were resolved in 8% SDS/PAGE gel electrophoresis under reducing conditions according to Laemmli (1970). Protein load in each slot was 3 μg, and gels were stained with Coomassie Brilliant Blue R. 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).

Spectrometric analysis. Untreated BSA and glycation treatments (BSA-Glc, BSA-Gal and BSA-Lac at 60°C for 120 min) were sent to the Arizona Proteomics Consortium (Proteomic Services, University of Arizona, USA) for their molecular mass determination by MALDI-TOF. Mass spectra were

acquired using an Applied Biosystems Voyager DE-STR device (Framingham, MA, USA) operating a 337 nm nitrogen laser. Dry sample pellets were resuspended in 0.1% trifluoroacetic acid (TFA) to give a final concentration of 2 μg/μl. A 5 μl sample aliquot was mixed with an equal volume of a saturated α-cyano-4-hydroxy-cinnamic 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 kV.

In order to identify modified amino acids, glycated BSA was digested with trypsin and chymotrypsin. Digestions of modified BSA were done in a 1 mg/ml solution (Shevchenko *et al*., 1996). Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was performed on the digested samples. One microgram of the digest was injected onto a linear quadrupole ion trap ThermoFinnigan LTQ mass spectrometer (San Jose, CA, USA) equipped with a Michrom Paradigm MS4 HPLC, a SpectraSystems AS3000 autosampler, and a nanoelectrospray source. Peptides were eluted from a 15 cm pulled tip capillary column using a gradient of 0–65% solvent B (98% methanol/2% water/0.5% formic acid/0.01% trifluoroacetic acid) over a 60-min period at a flow rate of 350 nl/min. The LTQ electrospray positive mode spray voltage was set at 1.6 kV, and the capillary temperature at 180°C. Data scanning was performed by the Xcalibur v 1.4 software (Andon *et al*., 2002).

Lectin binding assays. Serial two-fold dilutions of each treatment (BSA-Glc, BSA-Gal and BSA-Lac for 30, 60, 120, 180 or 240 min at 60°C) containing from 5 μg/μl to 10 ng/μl of protein were prepared and applied to nitrocellulose. Membranes were blocked overnight in PBS-Tween (20 mM phosphate buffer, 0.15 M NaCl and 0.05% Tween 20, pH 7.2) containing 1.5% BSA. Membranes were overlaid with biotinylated lectin to a final concentration of 10 μg/ml. Following 2 h incubation at 25°C, membranes were washed four times with PBS-Tween, incubated for 2 h with streptavidin-peroxidase (diluted 1:1000), washed four times again and the color reaction developed using 3,3'diaminobenzidine. Glycoproteins from pig duodenal mucin and immunoglobulins of porcine serum were used as positive controls. *Lens culinaris, Griffonia simplicifolia* I and *Ricinus communis* I lectins were used for BSA-Glc, BSA-Gal and BSA-Lac, respectively.

RESULTS AND DISCUSSION

In this work we obtained glycoconjugates through the Maillard reaction between BSA amino groups and carbonyl groups of carbohydrates (glucose, galactose and lactose). The protein-carbohydrate conjugation was time-dependent, amino acid-specific and with the highest reactivity for galactose. Only BSA-lactose was recognized by specific lectins.

Available amino groups. Free amino groups react with carbonyl groups of sugars to cause glycation (Fayle & Gerrard, 2002), the remaining free amino groups were measured by the OPA method. The measuring principle is based on the formation of 1-alkylthio-2-alkylisoindoles generated by the reaction of amino groups with *ortho*-phthaldialdehyde in the presence of a thiol; the produced compound possesses maximum absorbance at 340 nm (Frister *et al*., 1988). BSA incubated with glucose, galactose or lactose for 30, 60, 120, 180 or 240 min at 60°C showed a reduced content of available amino groups relative to untreated BSA (Fig. 1). In addition, the longer the heating time, the less available amino groups were detected. The reactivity of galactose was higher than that of glucose or lactose under these conditions. This observation is in agreement with previous reports where the reactivity order for β-lactoglobulin glycation was ribose>arabinose>galactose>rhamnose>lactose (Chevalier *et al*., 2001a, 2002). However, this sequence is not strict and reactivity could differ depending on glycation conditions and amino group source (Badui, 1993).

Gel electrophoresis. Important differences in migration patterns of glycated BSA were observed in SDS/PAGE (Fig. 2). BSA-Glc, BSA-Gal and BSA-Lac bands migrated broader and slower than untreated BSA, indicating that glycated samples contain a range of protein molecules with different number of coupled sugar residues. Furthermore, BSA-Lac presented tighter bands than BSA with monosaccha-

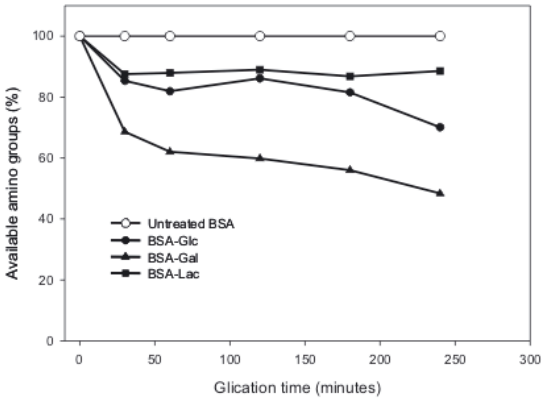

Figure 1. Available amino groups of untreated (without heating and carbohydrates) and glycated BSA with glucose, galactose and lactose at 60°C for 30, 60, 120, 180 or 240 min.

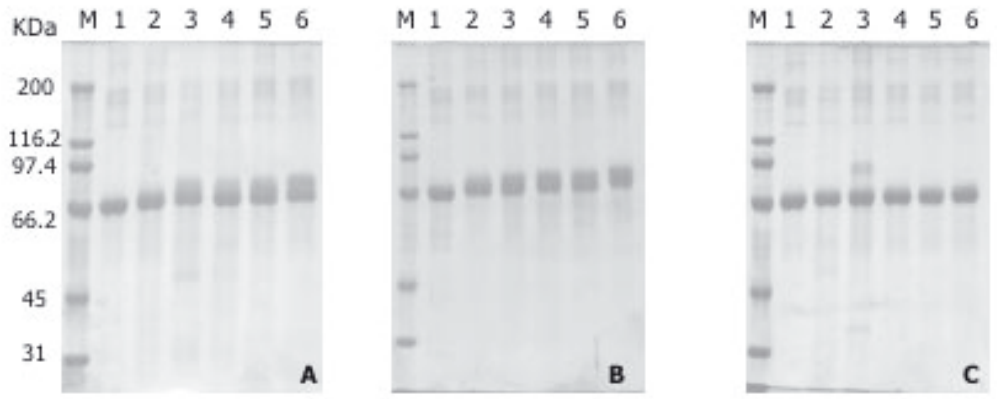

Figure 2. Electrophoretic analysis of glycated BSA.
SDS/PAGE in 8% gel of (1) untreated BSA (without heating and carbohydrates); (2–6) BSA heated at 60°C for 30, 60, 120, 180 or 240 min in presence of glucose (A), galactose (B) and lactose (C), respectively. M, molecular mass markers.

rides; the findings denoted that galactose and glucose were more reactive than lactose.

Spectrometry analysis. In this study, the confirmation of covalent addition of glucose, galactose and lactose to BSA was obtained by applying MALDI-TOF MS. Table 1 shows the molecular mass of the most abundant ion for each treatment. BSA-Gal presented the greatest increase (68104.2 Da) in mass, followed by BSA-Glc and BSA-Lac (66964.8 and 66951.1 Da, respectively). MALDI-TOF MS has been suggested as the most convenient method for neoglycan analysis; it is known to provide accurate molecular mass determination of proteins and glycoconjugates (Yeboah & Yaylayan, 2001).

As the condensation of a monosaccharide (either glucose or galactose) leads to a mass increase of 162 Da and of 324 Da for lactose, the number of carbohydrate molecules added was calculated by comparing the mass difference between glycated and untreated protein. Analysis of BSA conjugates with glucose, galactose and lactose for 120 minutes at 60°C showed 3.3, 10.3 and 1.6 molecules of carbohydrate added, respectively (Table 1). These data confirm that, under our experimental conditions, galactose appears to be the most reactive carbohydrate and is in agreement with the amino groups availability quantification described before.

Table 1. Mass values and glycation degree obtained from untreated and glycated BSA at 60°C for 120 min.

Treatment	Molecular mass average (Da)	Carbohydrate added[1] (molecules)
Untreated BSA	66431.3	–
BSA-Glc	66964.8	3.3
BSA-Gal	68104.2	10.3
BSA-Lac	66951.1	1.6

[1]To determine number of carbohydrate molecules added, mass difference (glycated – untreated BSA) was divided by 162 for glucose or galactose or 324 for lactose treatment.

In order to identify which amino-acid residues were modified, tryptic and chymotryptic digestions of glycated BSA were done. Seven tryptic and three chymotryptic modified peptides were generated from BSA-Gal. In all glycopeptides, the carbohydrate was coupled to lysine (Table 2). BSA-Glc treatment showed only two modified peptides and none for BSA-Lac. Even though it was expected to find three and one modified peptides for BSA-Glc and BSA-Lac, respectively, probably the ions were not sufficiently abundant to detect (relative to other peptides co-eluting with them). Moreover, some glycated peptides could display the same mass as unmodified ones. Besides, it has been reported that during glycation, fragmentation and dehydration of carbohydrates coupled to peptides can be observed, which can interfere with the identification of these glycopeptides (Stefanowicz *et al.*, 2001; Frolov *et al.*, 2006).

Even though BSA contains more lysines than those found modified, our results showed that lysines (K) at positions 117, 140, 256, 285, 297, 346, 374, 420, 523 and 597 were available for Maillard reaction when galactose was used. Also, some lysines appear either more exposed or in a more reactive environment, as observed for K256 and K420, since both were modified with galactose and glucose (Table 2). No modified Arg residues in BSA were detected. With some limitations, protease digestion followed by LC-MS was a useful tool in determining glycation sites, since different patterns of hydrolysis can be obtained for glycated and nonglycated samples (Chevalier *et al.*, 2001a; Lapolla *et al.*, 2004). Trypsin is a good protease choice since it cleaves proteins at the carboxyl side of amino acids lysine and arginine, and glycation at those amino-acid residues inhibit hydrolysis, leaving modified peptides.

Nacharaju and Acharya (1992) found that during hemoglobin glycation (pH 7.4 and 24°C for 3 h),

Table 2. Mass values of glycopeptides obtained by trypsin and chymotrypsin digestion of BSA-Gal and BSA-Glc glycated at 60°C for 120 min

Peptide	Sequence[1]	Mass (Da)	Position	Modified amino acid
BSA-Glc glycopeptides				
1	AEFVEVTK*LVTDLTK	1983.03	249–263	^{256}K
2	QNCDQFEK*LGEYGFQNALIVR	2692.75	413–433	^{420}K
BSA-Gal glycopeptides[2]				
1	ETYGDMADCCEK*QEPER	2295.78	106–122	^{117}K
2	LK*PDPNTLCDEFK	1738.75	139–151	^{140}K
3	AEFVEVTK*LVTDLTK	1983.03	249–263	^{256}K
4	ADLAK*YICDNQDTISSK	2105.07	281–297	^{285}K
5	NYQEAK*DAFLGSFLYEYSR	2464.48	341–359	^{346}K
6	LAK*EYEATLEECCAK	1977.94	372–386	^{374}K
7	QNCDQFEK*LGEYGFQNALIVR	2692.75	413–433	^{420}K
8	SALTPDETYVPK*AF	1538.72	512–525	^{523}K
9	ACDNQDTIAAK*L	1424.40	287–298	^{297}K
10	AVEGPK*LVVSTQTALA	1745.85	592–607	^{597}K

BSA-Lac glycopeptides; Unable to identify glycopeptides; [1]The modified K residues with glucose or galactose (+162 Da) are represented by K*. [2]The first seven peptides were generated by trypsin digestion and the last three by chymotrypsin digestion.

the availability of amino acids to join carbohydrates is a consequence of its three-dimensional structure rather than the amino-acid sequence around the glycation site. Additionally, glycation conditions and accessibility of the amino acids involved in the reaction contribute to the different sites and glycation degrees. Lapolla *et al.* (2004) established that *in vitro,* under conditions similar to physiological, human serum albumin glycation by glucose occurred in privileged sites K233, K276, K378, K525 and K545, which is in agreement with the fractional solvent accessible surface values calculated by molecular modeling. Although the present study conditions were different (longer incubation time and higher temperature), BSA molecular modeling (not shown) suggests that the modified amino acids were also the most exposed to the solvent on the protein surface.

Similar findings, of only K being modified, were reported for a casein-lactose system (Scaloni *et al.,* 2002). In contrast, Tagami *et al.* (2000) studying lysozyme glycation by glucose reported modification on both lysyl and arginyl residues.

Lectin binding assays. Sarabia-Sainz *et al.* (2006) previously reported that glycoconjugates obtained by Maillard reaction could be used as a strategy for biological recognition where the adhesin of *E. coli* K88 recognizes the conjugate modified with lactose. This is important since *E. coli* strains that bear K88 adhesin are the major cause of post-weaning diarrhea in piglets, thus glycoconjugates could protect against infections by blocking adhesion of lectin-carrying bacteria (Sharon, 2006). BSA-Glc, BSA-Gal and BSA-Lac glycoconjugates obtained according to Maillard reaction were evaluated for their recognition by specific lectins. Only the BSA-Lac conjugates were recognized by *Ricinus communis* I lectin, a β-galactose-specific lectin. Stronger interaction was observed with longer glycation time (Fig. 3). Lactose glycation involves coupling of the protein through glucose (reducing end), leaving galactose available for biological recognition (Boratyński & Roy, 1998). In contrast, BSA-Gal and BSA-Glc did not interact with either galactose- or glucose-specific lectins, *Griffonia simplicifolia* I and *Lens culinaris* (not shown).

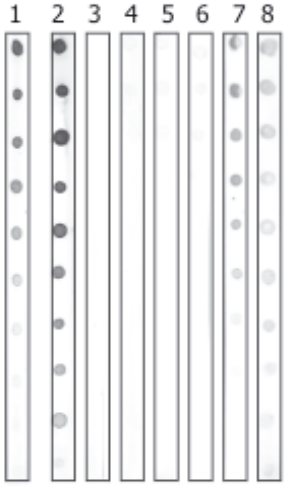

Figure 3. Recognition of BSA-Lac conjugates by *Ricinus communis* I lectin.
Glycoproteins (mucins and Ig's) and glycated BSA-Lac were applied in serial dilution (5 μg to 10 ng). Glycoproteins from pig duodenal mucins (1) and immunoglobulins of porcine serum (2) were used as positive controls. Untreated BSA (3), BSA-Lac at 60°C for 30 (4), 60 (5), 120 (6), 180 (7) or 240 min (8).

Aring *et al.* (1989) reported similar findings with serum albumin glycated by glucose, galactose or lactose using galactose/glucose-specific liver lectin receptor; only albumin-lactose was recognized by the receptor. In contrast, synthetically prepared thioglucose and thiogalactose albumin conjugates, where the hemiacetal ring structure is conserved, bind to the galactose/glucose specific liver lectins (Stowell & Lee, 1978). Thus, monosaccharides conjugated to proteins appear to produce acyclic forms not recognized by soluble or membrane bound lectins; special attention is required if biorecognition of the conjugate formed is required.

In conclusion, it was possible to obtain glycoconjugates by the chemical reaction between amino groups of bovine serum albumin and glucose, galactose or lactose. Under the conditions used in this study, galactose was more reactive than glucose or lactose. Chemical modification was observed by slower migration of glycated forms (SDS/PAGE) and a decrease of available amino groups. The increase in molecular mass and location of most modified amino acids were confirmed by mass spectrometry of glycated and protease-treated BSA. Only the highly solvent-exposed lysines were modified. Lysines 256 and 420 appeared the most available for conjugation. Even though it was possible to modify protein lysines with glucose, galactose and lactose, only BSA-lactose showed recognition by specific lectins.

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