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A BIOPHYSICAL COMPARISON OF HUMAN SERUM ALBUMIN TO BE GLYCATED *IN VIVO* AND *IN VITRO*

BIOFIZIČKO POREĐENJE ALBUMINA U HUMANOM SERUMU PODVRGNUTOG GLIKACIJI *IN VIVO* I *IN VITRO*

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Summary: The glycation process includes the arrangement of proteins with chemically reversible early glycation products, Schiff bases and Amadori adducts. These early products endure slow and complex rearrangements to create advanced glycation end-products (AGEs) that are involved in diabetic complications. Here, the biophysical characteristics of *in vitro* glycated human serum albumin (HSA) are compared to those of HSA glycated *in vivo*. The changes in the content of α-helices, AGE-specific fluorescence intensity, extent of lysine residue modification, and surface tension value and also the formation of Amadori products in HSA are similar in both conditions. It was observed, however, that arginine residues were modified only under physiological conditions (*in vivo*), while the same did not occur *in vitro*. This difference was related to the presence of 3-deoxyglucosone, a 1,2-dicarbonyl compound derived from glucose under physiological conditions. Therefore, the biophysical studies on the HSA glycation process *in vitro* are credible.

Keywords: human serum albumin, glycation, diabetic individual, arginine residue

Kratak sadržaj: Proces glikacije podrazumeva spajanje proteina sa hemijski reverzibilnim ranim proizvodima glikacije, Schiffovim bazama i Amadori aduktima. Spora i složena preraspodela tih ranih proizvoda vodi stvaranju krajnjih produkata glikacije (AGEs) koji utiču na komplikacije dijabetesa. Ovde su upoređene biofizičke karakteristike *in vitro* i *in vivo* glikozilisanog albumina u humanom serumu (HSA). Promene u sadržaju α-heliksa, intenzitetu fluorescencije specifične za AGE, obimu modifikacije lizinskog ostatka i vrednosti površinskog napona, kao i formiranje Amadori produkata u HSA slični su u *in vitro* i *in vivo* uslovima. Primećeno je, međutim, da su argininski ostaci pretrpeli modifikacije samo u fiziološkim uslovima (*in vivo*), ali ne i *in vitro*. Ta razlika odnosi se na prisustvo 3-deoksi-glukozona, 1,2-dikarbonilnog jedinjenja koje nastaje iz glukoze u fiziološkim uslovima. Zaključujemo da su rezultati biofizičkih ispitivanja procesa glikacije HSA *in vitro* verodostojni.

Ključne reči: albumin u humanom serumu, glikacija, dijabetičari, argininski ostatak

Introduction

Nonenzymatic protein glycation, as a multi-step chemical process, is the first event of hyperglycemia in biological fluids as well as tissues. This process is pathophysiologically important in the etiology of the long-term complications of diabetes. It involves the reaction of glucose with a number of amino acid side-chains bearing nucleophilic groups (such as the ε-amino groups of lysine residues and the N-terminal group of proteins). Accordingly, it finally yields a class of heterogeneous chemical compounds collectively referred to as advanced glycation end-products (AGE). The yield of these reactions depends on the glucose concentration and the resultant AGE can permanently alter protein structure and function (1–4). The glycated proteins accumulate in diabetic patients more than in normal subjects because of the presence of high glucose concentration during diabetes (5). In principle, the functions of glycated proteins differ from those of their non-glycated form. This fact has been suggested for human

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serum albumin (HSA), hemoglobin, and a series of intracellular enzyme proteins (6).

HSA is the most abundant (40 mg/mL) and quantitatively the most important depot and transport protein in blood plasma. It is a major antioxidant with important roles in maintaining normal osmolarity of plasma and interstitial fluids. It is synthesized in the liver as a single, non-glycosylated polypeptide, which is organized to form a heart-shaped protein comprising about 67% α-helix with no β-sheet (7–9). HSA glycation is of special interest because HSA bears approximately 58 Lys residues, making it a favorable target for the glycation process (10). The identification of the sites of glycation, structural and functional changes during glycation of HSA, and AGE formation in the presence of different carbohydrates have been the subject of recent *in vitro* studies (11–18). The level of glycated albumin might also be of value as an indicator of the degree of hyperglycemia in diabetics (12).

We have reported studies about the glycation of HSA *in vitro* and reported its characteristics and structural changes during the glycation process (19–21). In this study, the aim was to compare the structual characteristics of *in vitro* glycated human serum albumin with those of its counterpart in human serum.

Materials and Methods

Materials

HSA, β-D (+) glucose, p-nitroblue tetrazolium-chloride (NBT), Cibacron Blue-3GA Agarose and millipore ultrafiltration membrane (30 KDa) were from Sigma. The membrane filter with 0.2 μm pore size (25 mm in diameter) and dialysis tubing of 10,000 MW cut-off were from Whatmann (UK).

Sodium azide, EDTA, 9,10-phenanthrenequinone were from Merck (Germany). 2,4,6-trinitrobenzene sulfonic acid (TNBSA) was from Fluka. All other materials were of analytical grade. All solutions were prepared with deionized water.

Methods

In vitro glycated HSA was obtained by incubation of HSA with different concentrations of glucose (4.95–22.0 mmol/L) in sodium phosphate buffer (50 mmol/L Na_2HPO_4/NaH_2PO_4 pH 7.4, 1 mmol/L EDTA, and 0.1 mmol/L sodium azide) in capped vials under sterile conditions at 37 °C in the dark for 14 and 28 days. Glucose concentrations selected were similar to the glucose concentration of normal and diabetic individuals. Samples were used for further studies after being dialyzed against sodium phosphate buffer at 4 °C for 48 h.

In vivo glycated HSA were obtained from a pool serum of healthy and diabetic volunteers. Serum samples were filtered with a Millipore ultrafiltration membrane (cut-off: 30 kDa, using an Amicon Ultrafiltration apparatus) followed by affinity column chromatography (1*20 cm) on Agarose-Immobilized Cibacron Blue 3GA (22–23). The absorbance of eluent solution at 280 nm was recorded with a Shimadzu spectrophotometer, model UV-3100. For the investigation of protein separation and purity, SDS-PAGE was carried out according to the method of Laemmli (24). Protein bands were detected by a Coomassie staining method (25). Concentrations of protein samples were determined in triplicate by the bicinchoninic acid protein assay (BCA assay).

The details of determination of the modification extent of side-chain residues (Lysine (12) and Arginine (26)) and Amadori product extent (26), measurements of AGEs fluorescence (19, 26), surface tension (21) and Far-UV CD (19) reported in this work have been previously described in detail.

Results

Many studies discussed the structural changes and characteristics of proteins during the glycation process *in vitro* (11–21, 27), but the compatibility and confirmation of these properties with what occurs *in vivo* have not been approached. In order to determine whether the results of structural changes and characteristics of glycated albumin *in vitro* are compatible with those in the serum of diabetic patients, purification of albumin from the pool serum of normal and diabetic patients with different glucose blood concentrations was performed. The concentrations of glucose in the human serum samples are listed in *Table I*. Albumin was purified by a Cibacron Blue-3GA Agarose column and the absorbance of eluent solution at 280 nm is shown in *Figure 1A*. Previous studies showed that this approach is very effective to remove albumin from human plasma (22, 23). After purification, SDS-PAGE and Coomassie staining methods were used to confirm the purification method (*Figure 1B*). Based on the result, HSA was truly purified from other proteins present in the samples. Also, HSA was incubated with different concentrations of glucose (similar to *in vivo* conditions) for 14 and 28 days to study the progression of glycation reaction *in vitro*. Afterwards, experiments

Table I The concentration range of sugar in human serum samples of normal and diabetic individuals.

Sample	Serum sugar concentration (mmol/L)	Condition
1	< 5.5	Normal individual
2	8.25–11.0	Diabetic individual
3	11.0–13.5	Diabetic individual
4	16.5–19.25	Diabetic individual
5	> 22.0	Diabetic individual

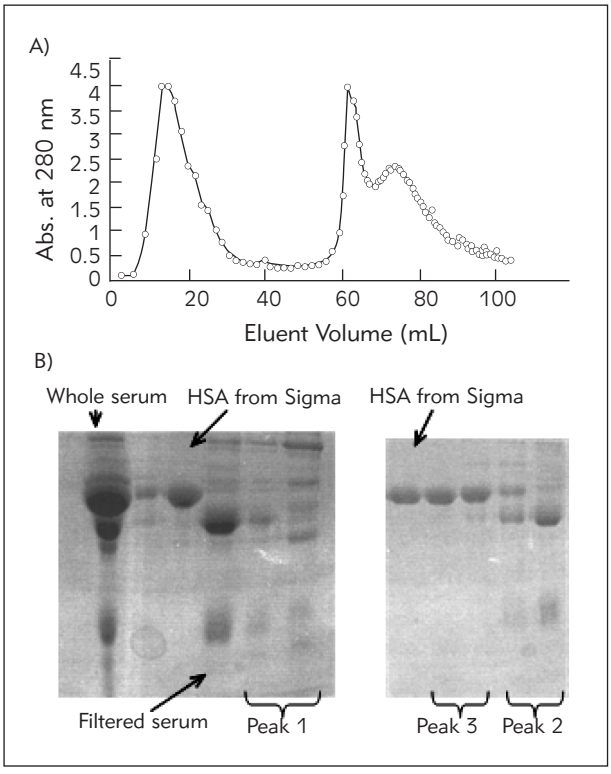

Figure 1 A) A typical affinity chromatogram for filtered pool diabetic serum on a Cibacron Blue-3GA Agarose column (1*20 cm). The protein fractions were measured at 280 nm. B) The results obtained from the SDS PAGE of the samples of eluted solution of filtered pool diabetic serum from the location 1, 2, 3 from chromatogram and control HSA from Sigma.

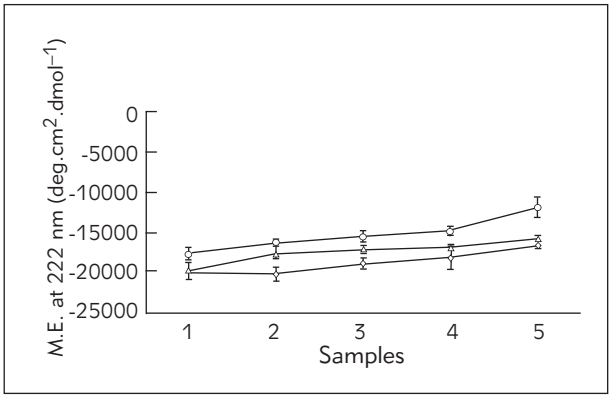

Figure 2 Molar ellipticity at 222 nm for HSA samples which were purified from human serum (°) and *in vitro* glycated samples (△, ◊) after 14 days of incubation (△) and after 28 days of incubation (◊) in phosphate buffer, pH 7.4, with (1) < 5.5, (2) 8.25–11.0, (3) 11.0–13.5, (4) 16.5–19.25 and (5) > 22.0 mmol/L sugar concentration.

were designed as follows to discriminate the albumin glycation process *in vitro* and *in vivo*. To evaluate the changes in the secondary structure, the circular dichroism spectra (Far-UV CD) in the range of 190±260 nm were acquired for the albumin

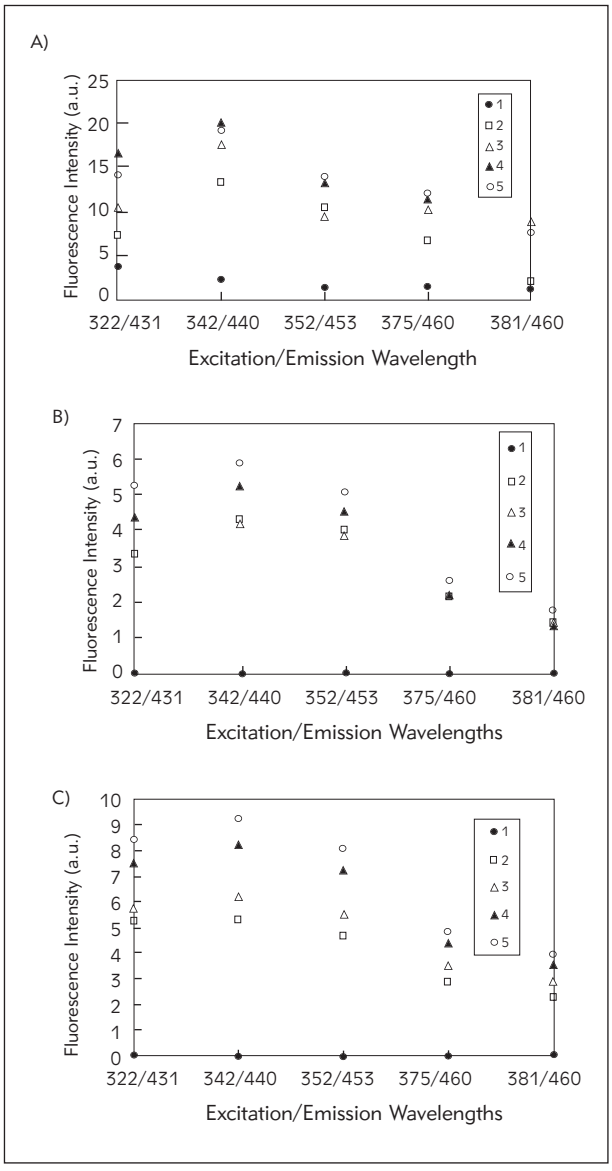

Figure 3 Fluorescence intensity ratio of excitation/emission at different wavelengths (322/431, 342/440, 352/453, 375/460, 381/460) for samples of HSA that were A) purified from human serum, B) *in vitro* glycated samples after 14 days of incubation and C) *in vitro* glycated samples after 28 days of incubation, in phosphate buffer, pH 7.4, with (1) < 5.5, (2) 8.25–11.0, (3) 11.0–13.5, (4) 16.5–19.25 and (5) > 22.0 mmol/L sugar concentration.

samples. Molar ellipticity at 222 nm shows changes in the helical structure of proteins. These changes for albumin samples are represented in *Figure 2*. In this figure, upon increasing sugar concentration in the serum of diabetic patients and *in vitro* samples (with respect to the HSA of normal glucose individuals), there is a decrease in the negative ellipticity at 222 nm. The result indicates a loss of helical structure.

Therefore, increasing the sugar concentration or the degree of hyperglycemia leads to a decrease in the content of α-helices of HSA, compared to normal

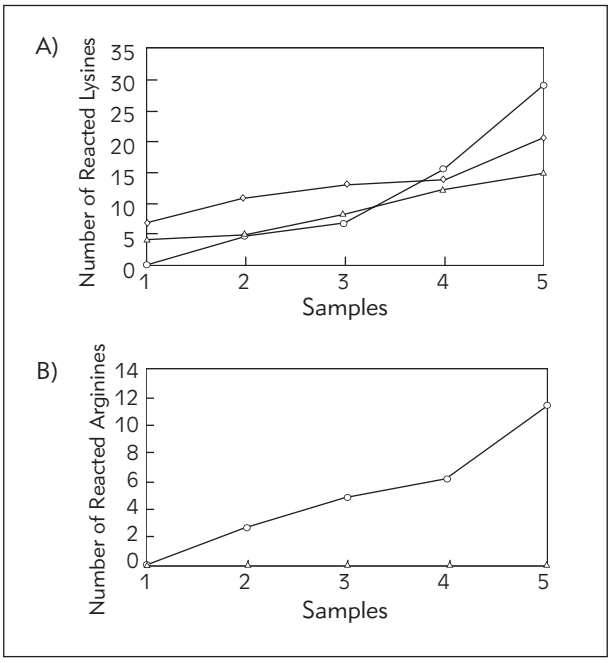

Figure 4 Number of reacted lysine residues, and B) arginine residues for HSA samples which were purified from human serum (°) and *in vitro* glycated samples (△, ◊) after 14 days of incubation (△) and after 28 days of incubation (◊) in phosphate buffer, pH 7.4, with (1) < 5.5, (2) 8.25–11.0, (3) 11.0–13.5, (4) 16.5–19.25 and (5) > 22.0 mmol/L sugar concentration.

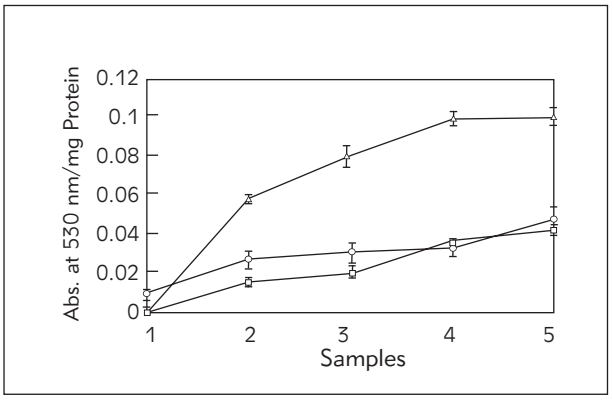

Figure 5 Amount of Amadori products formed per miligram of HSA for samples which were purified from human serum (°) and *in vitro* glycated samples (△, ◊) after 14 days of incubation (△) and after 28 days of incubation (◊) in phosphate buffer, pH 7.4, with (1) < 5.5, (2) 8.25–11.0, (3) 11.0–13.5, (4) 16.5–19.25 and (5) > 22.0 mmol/L sugar concentration.

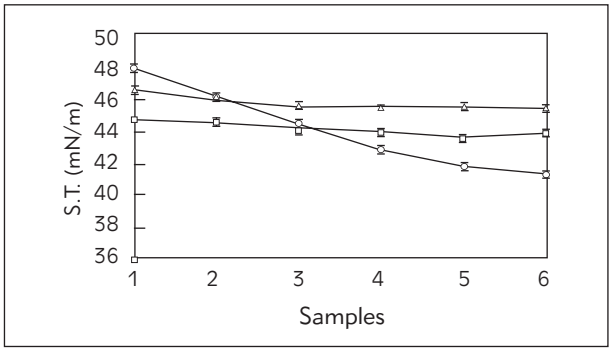

Figure 6 Surface tension of 1 mg/mL samples of HSA which were purified from human serum (°) and *in vitro* glycated samples (△, ◊) after 14 days of incubation (△) and after 28 days of incubation (◊) in phosphate buffer, pH 7.4, with (1) < 5.5, (2) 8.25–11.0, (3) 11.0–13.5, (4) 16.5–19.25 and (5) > 22.0 mmol/L sugar concentration.

individuals. Structural changes of glycated HSA to the β-structure and nanofibril formation have been reported previously (21).

In our previous *in vitro* study (19), it was shown that some AGEs are fluorescent with maximum emission wavelengths at 431, 440, 453, and 460 nm at correspondence excitation maximum of 322, 342, 352, 375 and 381 nm, respectively. Thus, all these five excitation/emission wavelengths were used for the detection of AGE fluorescence in purified HSA and *in vitro* samples. *Figure 3A* shows these alterations for purified albumin from normal and diabetic human serum. Also, *Figures 3B* and C show these alterations for *in vitro* glycated albumins after 14 and 28 days of incubation. As shown in these figures, AGE-specific fluorescence was found to increase, followed by increasing sugar concentration in the serum of diabetic patients in *in vitro* conditions. Another, more interesting method to follow the formation of AGE is the detection of lysine and arginine side-chain modifications. Extensive modification to lysine side-chains and minor modification to arginine side-chains have been found to occur during the AGE -formation process with glucose in albumin (26). Free lysine and arginine residues were measured by TNBSA (26) and 9,10-phenanthrenequinone (27) reagents, respectively. *Figure 4* shows the number of reacted lysine and arginine residues in each HSA molecule of the samples. The number of free lysine and arginine residues in diabetic patients decreased by increasing the level of glucose in serum, compared to the normal individual. Also, *Figure 4* shows a decrease in the level of free lysine residue in glycated albumin *in vitro*. However, the numbers of free arginine do not change in these *in vitro* samples. This study shows that in the sample of HSA incubated for 35 days in the presence of 27.5 mmol/L glucose, approximately 2 out of 25 arginine residues in HSA were modified. Also, the results show that in diabetic patients with a sugar concentration greater than 22.0 mmol/L, approximately 63% and 46% of lysine and arginine residues (*Figure 4*) are modified, respectively. Previous investigations showed that 3-deoxyglucosone, a 1,2-dicarbonyl compound derived from glucose, reacts with arginine in albumin under physiological conditions, whereas glucose itself reacts more extensively with lysine (26).

Another criterion for the glycation reaction is the generation of ketoamines called Amadori products, which is detected using a fructosamine assay (26). In this assay, Amadori products (ketoamines) can reduce NBT reagent and then produce colored formazan dye with an absorption maximum at 530 nm. In *Figure 5*, formation of formazon dye in purified HSA and *in vitro* samples in different glucose concentrations were compared. The result reflects the presence of Amadori products, the extent of which increases by increasing the level of glucose in serum and in the incubation medium.

During the glycation process, modification of protein structures by carbohydrates and the interaction of carbohydrates with proteins affect the surface rheology of globular proteins adsorbed at an air-water interface. *Figure 6* shows the values of surface tension of purified HSA (1 mg/mL) with different levels of sugar in serum (measurements were performed at 25 °C). The surface tension of 1 mg/mL HSA of a normal individual was approximately 48 mN/m. Increasing the level of sugar concentration in serum led to decrease in surface tension under two conditions. Glycation of lysine and arginine residues and perhaps other charged and hydrophilic amino acids may also result in an alteration in hydrophobicity/hydrophilicity of the surface of the protein and induce decreasing surface tension.

Based on the obtained results, a comparison of the characteristics of glycated HSA *in vitro* and those observed for glycated HSA *in vivo* indicates that structural changes of HSA in diabetic patients are compatible with those obtained in *in vitro* conditions. Increasing the sugar concentration or the degree of hyperglycemia leads to a decrease in the content of α-helices of HSA, level of free lysine residue and surface tension values and an increase in AGE-specific fluorescence intensity and the Amadori product formation. The only difference concerns the modification of arginine residues. Glucose reacts more extensively with lysine. However, compounds derived from glucose under physiological conditions: 3-deoxyglucosone and 1,2-dicarbonyl compounds, react with arginine residue *in vivo*.

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Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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