Supplementary Information

Glycation of human serum albumin alters its binding efficacy towards the dietary polyphenols: A comparative approach

Atanu Singha Roy^{1,2,*}, Pooja Ghosh¹ and Swagata Dasgupta¹

¹ Department of Chemistry, Indian Institute of Technology, Kharagpur, India

² Present address: Department of Chemistry, National Institute of Technology,
Meghalaya 793003, India

***Corresponding author:** Email: singharoyatanu@gmail.com, Tel.: (+91)-364-2501294,
Fax. (+91)- 364-2501113

1. Synthesis and characterization of the glycated HSA

1.1. Methodology

1.1.1. Preparation of glycated HSA

The glycated analogue of HSA was prepared *in vitro* according to the methodology of Joseph *et al.* 2011 (Joseph, Anguizola, & Hage, 2011). For the preparation of glycated sample we have followed the incubation of native HSA (0.59 mM) with 0.05 M of solid D-glucose in 0.1 M phosphate buffer of pH 7.4 at 37 °C. The autoclaved water is used for the solution preparation. Sodium azide (1 mM) was added to the above mixture to avoid the bacterial expansion during incubation time period. After that the whole mixture was incubated for one month time (30 days) duration at 37°C. Dialysis technique (repeated for three times) was used to remove the excess glucose content at 4°C using water (200-500 volumes of the sample). After this lyophilization the sample was stored at -20 °C for further study (Joseph, Anguizola, & Hage, 2011). The Bradford assay was carried out to

determine the concentration of glycated sample using BSA (1 mg/mL) as the standard (Bradford, 1976).

1.1.2. Measurements of carbonyl content

The 2,4-dinitrophenyl hydrazine (DNPH) assay was conducted to find out the glucose content of glycated samples (Reznick & Packer, 1994). Initially at room temperature 0.2 mL of glycated protein sample (4.29 mg/mL) was incubated for 1.5 h with 0.8 mL 0.1% DNPH in 2.5 M HCl solution. After that 1 ml 20% trichloroacetic acid (TCA) was added to the above mixture and incubated further on ice for half an hour to precipitate the protein. Then the mixture was centrifuged at 10000 rpm to form the pellet. The pellet was washed properly with ethyl acetate:water (50:50 v/v) mixture twice. Finally the pellet was resuspended with 0.5 M guanidine hydrochloride. The carbonyl content of the protein sample was calculated by measuring the absorbance of the mixture at 370 nm with the help of the molar extinction coefficient of the dinitrophenylhydrazone species: $\epsilon_{370} = 22000 \text{ M}^{-1} \text{ cm}^{-1}$ (Vetter & Indurthi, 2011).

1.1.3. MALDI-TOF measurements

The total mass of HSA and its glycated form were measured on a MALDI-TOF instrument (Voyager-De Pro, Applied Biosystems, USA). The matrix for the experiment was prepared by dissolving solid sinapinic acid (20 mg/mL) in water and HPLC grade acetonitrile (50:50 v/v) that contains 0.1% trifluoroacetic acid (TFA). The protein samples were mixed with the freshly prepared matrix (50:50 v/v) for the MALDI-TOF measurements. The proteins were then placed on the MALDI plates and kept back for 6

for complete drying. The scanning was executed using accelerating voltage of 25 kV. The mass acquirement range for this purpose was selected between 20 and 80 kDa.

The tryptic digestion was carried out to identify the modified amino acid residues in glycated protein samples. We have treated HSA and its glycated analogue in a same fashion for this experiment. In the very first step digestion buffer was prepared as follows: 10 mg of solid ammonium bicarbonate was dissolved in 2.5 mL of millipore water to obtain an ultimate concentration of 50 mM and the same was stored at 4 °C for further use. 8 mg of dithiothreitol (DTT) was dissolved in 0.5 mL of millipore water to make 100 mM and stored at -20 °C. Resultant concentrations of the protein samples were kept as 1 mg/mL. Alkylation buffer was prepared just before the use by dissolving 9 mg of iodoacetamide in 0.5 mL of millipore water and kept in the dark by wrapping with aluminium foil. Trypsin (0.1 µg/µL) solution for the experiment was arranged by dissolving 20 µg of solid in 0.2 mL of millipore water.

15 µL of the digestion buffer and 1.5 µL of the reducing buffer were taken in a 1 mL microcentrifuge tube and a 10 µL of the sample was just added to the above mixture and volume was accustomed to 30 µL. The resultant solution was then incubated at 95 °C for 5 minutes and permitted to cool down at room temperature.

Then a 3 µL of the alkylation buffer was appended to the above solution and incubated further in a dark place for 25 minutes. Finally 1 µL trypsin solution was mixed to the above resultant solution and kept at 37 °C for 3 h. Another 2 µL trypsin solution was added to the above mixture and incubated at 37 °C for overnight.

The α -cyano-4-hydroxycinnamic acid was chosen as the matrix for MALDI-TOF measurements. 1 μ L digested protein sample was spotted on the Maldi sample plate followed by 1 μ L of matrix. The spots were allowed to dry completely and after that MALDI data were collected.

1.1.4. Circular dichroism (CD) measurements

CD experiments were performed on a Jasco-810 spectropolarimeter using a path length of 0.1 cm at room temperature. Far UV CD spectra were collected in the range of 190-240 nm with a scan speed of 50 nm/min and a response time of 4 s. Baseline corrections and blank spectra subtractions were carried out according to the necessitate of the experiments.

1. 2. Characterizations

1.2.1. UV-vis and fluorescence spectroscopy

The natural browning was noticed in open eye for the glucose modified samples after 30 days incubation. After the dialysis no precipitation of HSA was observed which indicated that the protein can uphold glycation without any loss of its solubility. Generally a visible browning was noticed for the glucose customized samples which indicates the association of Maillard reaction (Maillard, 1912). The UV-vis spectrum of the glycated sample (Figure S1a) was found analogous to glucose modified bovine serum albumin (Vetter and Indurthi, 2011) and dihydroxy acetone modified human serum albumin (Ahmed *et al.* 2013). But the glycated sample exhibited the characteristics peak of HSA at 280 nm with a hyperchromicity of 10%. The modified HSA also showed a significant drop in the

tryptophan fluorescence intensity ($\sim 58\%$) (Figure S1b) similar to the study reported previously by Coussons *et al.* in 1997 (Coussons, 1997).

The existence of different advanced glycation end products (AGEs) was characterized by fluorescence spectroscopy. The presence of argpyrimidine, pentosidine and malonaldehyde were detected by recording their emissions at 410, 430 and 450 nm respectively (Figure S2). Figure S3 represents the fluorescence emission intensities of different other AGEs at 430 nm upon excitation at 350 nm at different time intervals.

Figure S1: (a) UV-vis spectra of HSA (brown line) and glycated HSA (green line) in 20 mM phosphate buffer of pH 7.0. [Protein] = $10\ \mu\text{M}$. (b) Fluorescence emission spectra of native HSA (brown line) and glycated HSA (green line). $\lambda_{\text{ex}} = 295\ \text{nm}$ and [Protein] = $2\ \mu\text{M}$.

Figure S2: Fluorescence emission spectra of formation of (a) argimidine, (b) pentosidine, (c) malonaldehyde and (d) other AGEs after the glycation over 30 days. λ_{ex} = 320, 335, 370 and 350 nm for (a), (b), (c) and (d) respectively. [Protein] = 2 μ M.

Figure S3: Fluorescence intensities of HSA incubated with 50 mM of D-glucose at different time intervals.

1.2.2. Carbonyl content from DNPH assay

The degree of structural modification was estimated by using DNPH assay where the breaking of the glycation end products is conducted in strong acidic medium. The carbonyl content in the glycated HSA was found to be $(5.75 \pm 0.014) \mu\text{M}/\mu\text{M}$ of the protein and it is the indicative of an advanced stage of diabetes.

1.2.3. MALDI-TOF results

Figure S4a stands for the MALDI-TOF spectra of native HSA and glycated analogue of HSA respectively. Native HSA exhibited a wide band centered at the m/z of 66475.10 Da and the glycated HSA displayed a narrow band centered at the m/z of 67563.73 Da. It has been observed that six glucose molecules are attached covalently to the protein molecule.

Figure S4a: MALDI-TOF spectra of the native and glycated HSA

To examine the probable glycation sites in HSA the native and the glycated samples were treated independently with trypsin and the tryptic digestion products were judged by MALDI-TOF mass spectrometer. The theoretical consequences of tryptic digestion products of HSA were used to compare with the experimental findings. Residues like lysine (Lys), arginine (Arg) and cysteine (Cys) due to their nucleophilic characteristics are accountable to glycation under different experimental conditions. Literatures indicate that Lys 525 is the major glycation site along with Lys 525, Lys 199, 281 and 489 that play a significant contribution to glycation (Garlick & Mazer, 1983; Shaklai, Garlick, & Bunn, 1984; Iberg & Fluckiger, 1986).

The mass of tryptic fragments of the glycated sample have been listed in Table S1. The identification of the fragments is done as follows:

- (i) One HSA fragment having a mass of m/z of 1230 indicates the sequence 11-20 and after addition of a glucose molecule the m/z changes to 1398.
- (ii) Another modified peptide fragment bearing an m/z of 1766 is formed from an m/z of 1591 of the peptide sequence (190-209) of native HSA which indicated the possible modification of Lys 199 or Lys 205 of the sequence.
- (iii) Residues Lys 159, 233 and 525 were also modified as obtained from tryptic digestions.
- (iv) One arginine residue (Arg 160) was modified in the sequence 160-181 exhibiting an m/z of 2713 from an m/z of 2486 of HSA.

The occurrence of argpyrimidine was detected from the fluorescence behaviour of the sample at 320 nm and monitoring the emission at 410 nm. The present results were found

analogous to the previously reported literatures. (Wa, Cerny, Clarke & Hage, 2007; Frost, Chaudhry, Bell & Cohenford, 2011).

1.2.4. CD results

The negative bands around 208 and 222 nm are the characteristics of α -helical configuration of HSA. The incubation of HSA with D-glucose for 30 days results in the reduction of α -helical content of native HSA from 55 to 48% and the corresponding β -sheet content increased from ~ 5 to ~ 10 % as estimated from DICHROWEB software (Whitmore & Wallace, 2004). It has also been observed that the polyphenols quercetin, rutin and genistein reduced the helical content of the glycated HSA (Table S3, Figure S7b). On the other hand morin and fisetin were found to increase the %-helicity (Table S3, Figure S7c) of the modified protein. The decrease in % α -helicity is observed may be due to partial unfolding of protein structure as observed in case of binding of genistein and curcumin with native HSA (Mandeville, Froehlich & Tajmir-Riahi, 2009).

Figure S5: UV-vis spectra of different polyphenols (20 μM) in absence (brown line) and presence of the glycated HSA (0 to 25 μM) in 20 mM phosphate buffer of pH 7.0. The Benesi-Hildebrand plots for the interactions of polyphenols with glycated HSA.

Figure S6: UV-vis spectra of glycated HSA, different polyphenols and polyphenol-protein complexes (difference spectra of protein-ligand and ligand) in 20 mM phosphate buffer of pH 7.0.

Figure S7. (a) CD spectra of the native HSA (brown line) and glycosylated HSA (green line). HSA was incubated with D-glucose for 30 days in 20 mM phosphate buffer of pH 7.0. (b) and (c) CD spectra of glycosylated HSA in absence (black line) and presence of polyphenols (4 μ M, color lines) in the experimental buffer. [HSA] = [glycosylated HSA] = 2 μ M.

References

Ahmed, S., Shahab, U., Baig, M. H., Khan, M. S., Salaman Khan, M., Srivastava, A.K., Saeed, M., & Moinuddin (2013). Inhibitory Effect of Metformin and Pyridoxamine in the Formation of Early, Intermediate and Advanced Glycation End-Products. *PLoS ONE* 8, e72128. doi: 10.1371/journal.pone.0072128

Bradford, M. M. (1976). Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248-254. doi: 10.1016/0003-2697(76)90527-3

Coussons, P. J., Jacoby, J., McKay, A., Kelly, S. M., Price, N. C., & Hunt, J. V. (1997). Glucose modification of human serum albumin: a structural study. *Free Radic. Biol. Med.* 22, 1217–1227. doi: 10.1016/S0891-5849(96)00557-6

Frost, L., Chaudhry, M., Bell, T., & Cohenford, M. (2011). In vitro galactation of human serum albumin: Analysis of the protein's galactation sites by mass spectrometry. *Anal. Biochem.* 410, 248–256. doi: 10.1016/j.ab.2010.11.034

Garlick, R. L., & Mazer, J. S. (1983). The principal site of nonenzymatic glycosylation of human serum albumin in vivo. *J. Biol. Chem.* 258, 6142–6146. Retrieved from: <http://www.jbc.org/content/258/10/6142.long>

Iberg, N., & Fluckiger, R. (1986). Nonenzymatic glycosylation of albumin in vivo. Identification of multiple glycosylated sites. *J. Biol. Chem.* 261, 13542–13545. Retrieved from: <http://www.jbc.org/content/261/29/13542.long>

Joseph, K. S., Anguizola, J., & Hage, D. S. (2011). Binding of tolbutamide to glycated human serum albumin. *J. Pharm. Biomed. Anal.* 54, 426–432. doi: 10.1016/j.jpba.2010.09.003

Maillard, L. C. (1912). Action of Amino Acids on Sugars. Formation of Melanoidins in a Methodical Way, *Compt. Rend.* 154, 66–68.

Mandeville, J. S., Froehlich, E., & Tajmir-Riahi, H. A. (2009). Study of curcumin and genistein interactions with human serum albumin. *J. Pharm. Biomed. Anal.* 49, 468–474. doi:10.1016/j.jpba.2008.11.035

Reznick, A. Z., & Packer L. (1994). Oxidative damage to proteins: spectrophotometric method for carbonyl assay. *Methods Enzymol.* 233, 357–363. doi:10.1016/S0076-6879(94)33041-7

Shaklai, N. Garlick, R. L., & Bunn, H. F. (1984). Nonenzymatic glycosylation of human serum albumin alters its conformation and function. *J. Biol. Chem.* 259, 3812–3817. Retrieved from: <http://www.jbc.org/content/259/6/3812.long>

Vetter, S. W., & Indurthi, V. S. K. (2011). Moderate glycation of serum albumin affects folding, stability, and ligand binding. *Clin. Chim. Acta* 412, 2105–2116. doi:10.1016/j.cca.2011.07.022

Wa, C., Cerny, R. L., Clarke, W. A., & Hage, D. S. (2007). Characterization of glycation adducts on human serum albumin by matrix-assisted laser desorption/ ionization time-of-flight mass spectrometry. *Clin. Chim. Acta* 385, 48–60. doi:10.1016/j.cca.2007.06.011

Whitmore, L.; Wallace, & B. A. (2004). DICHROWEB, an online server for protein secondary structure analyses from circular dichroism spectroscopic data. *Nucleic Acids Res.* 32, W668–W673. doi: 10.1093/nar/gkh371

Table S1. Glycated peptide fragments identified from tryptic digestions by MALDI-TOF analyses

Peptide (aa)	Theoretical mass of the fragments (Da)	+ One glucose (Da)	Mass observed (Da)	Analysis
11-20	1230	1410	1398	Lys 12
145-160	1760	1926	1961	Lys 159
160-181	2486	2688	2713	Arg 160
196-209	1591	1770	1766	Lys 199 or Lys205
226-240	1647	1830	1838	Lys 233
525-534	1160	1340	1335	Lys 525

Table S2. Ground state association constants for the interactions of the polyphenols with the glycated HSA

Ligands	$10^{-4} \times K_a (M^{-1})$	Red Shift (nm)
Quercetin	8.96±1.25	25
Rutin	1.89±0.05	4
Morin	6.59±0.56	10
Fisetin	7.27±0.83	20
Genistein	7.04±0.98	6

The $\pm$ values after the numeric represent a range of ± 1 S.D.

Table S3. The α -helical content of native HSA after glycation and the α -helical content of glycated HSA (gHSA) after polyphenol binding

System	% α -helix
Native HSA	54.9 $\pm$ 2.25
Glycated HSA	48.1 $\pm$ 0.55
Quercetin/gHSA	46.3 $\pm$ 2.65
Rutin/gHSA	44.7 $\pm$ 0.42
Genistein/gHSA	45.2 $\pm$ 1.76
Morin/gHSA	53.1 $\pm$ 1.55
Fisetin/gHSA	54.3 $\pm$ 2.12

The $\pm$ values after the numeric represent a range of $\pm$ 1 S.D.