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RESEARCH ARTICLE

CHARACTERIZATION OF GLYCATION PATTERN AND RELATED POST TRANSLATION MODIFICATIONS ON HUMAN SERUM ALBUMIN

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ABSTRACT

Due to excessive glycation, structure of protein may get altered, which results in abnormal functions of proteins. The glycation process can occur inside or outside of the body, whenever glucose molecules react with proteins. The impaired proteins are named as advanced glycation end products (AGEs). The AGEs are responsible for various diabetic complications and may lead to various diseases. AGEs can block the insulin receptors on cells and patient may develop insulin resistance. Glycation affects all cells, making it responsible for aging. Formation of unstable Schiff's base is first step in glycation reaction followed by conversion of Schiff's base into stable reversible Admori product in second step. Finally, AGEs are formed by further changes in Admori products by series of reactions of dehydration and fragmentation. Glycation reaction mainly occurs at amino group of lysine and arginine residues, as glycation process involves proteins, it becomes indispensable to study proteins. This study was carried out to know the positions of amino acids which are always accessible under different conformational changes, for smaller molecules like glucose. Due to which chances of glycation at specific positions increases where numbers of post translation modifications are possible. Various studies regarding the specific sites of glycation on human serum albumin are carried out all over the world but these studies were restricted to sites of crystal structure of human serum albumin alone or sites of glycated human serum albumin glycated with glucose and similar glycating agent. Due to conformational change of human serum albumin after binding of another ligand the positions of glycation changes because of this glycation pattern has direct relation with the structural conformation of protein. So, we have studied possible conformations of human serum albumin, The crystallographic structure of human serum albumin was obtained from the Protein Data Bank (PDB), a repository for the 3-D structural data of large biological molecules, such as proteins. We have analysed changes in accessible positions of lysine and arginine on HSA after under different conformational changes due to ligand binding. Finally, we have analysed the glycated sample of human serum albumin by liquid chromatography-mass spectrometry (LC-MS) to study post translation modifications on identified glycation prone lysine and arginine amino acid positions of human serum albumin.

Key words: Glycation, human serum albumin, liquid chromatography-mass spectrometry, accessibility of amino acids, Swiss-PDB Viewer.

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INTRODUCTION

The reaction of sugar with protein is non-enzymatic in nature (Maillard, 1912), leading to undesired modifications of carbohydrates. In early-stage glycation free primary amino groups are involved (Lapolla *et al*., 2005). The lysine and arginine are mainly involved in such reactions leading to the formation of Schiff's base (Zhang *et al*., 2009), which is an unstable intermediate. Schiff base further forms stable Amadori products (Monnier, 1990). The Amadori products undergo various reactions such as cross-linking, oxidation to form AGEs. The AGEs can be found on N-terminus alongside lysine and arginine (Kislinger *et al*., 2004). The AGEs formed during diabetes can cause chronic vascular complications.

The AGEs can be endogenous as well as exogenous in disease states (Vlassara, 2005). Over a period of time due to the accumulation of AGEs there is an increase in the severity of diabetes and other diseases (Brownlee, 2000). The AGEs can also be correlated with aging and age-related diseases such as Alzheimer's disease and cancer (Wells-Knecht *et al*., 1995; Vitek *et al*., 1994). D-glucose, ribose interacts with protein for non-enzymatic glycation (Neglia *et al*., 1983). The final step, in the cascade of glycation reactions, is the formation of AGEs (Reynolds, 1965). Uncontrolled sugar level and related formation of AGEs are deciding factors for diabetes-related problems (Thornalley, 2003a; Goldin *et al*., 2006). The glycation of lysine and arginine, changes the structure and function of the protein (Thornalley *et al*., 2003b).

Surface accessibility is the deciding factor in the glycation of protein (Quan *et al*., 1999). In this study, the major focus is on the glycation of the most abundant protein i.e. human serum albumin (HSA). In the recent few decades, there is more focus on research of HSA. The study of structural changes can be useful in determining the accessibility of lysine and arginine for glycation reaction. Many glycation sites were previously reported, which shows higher glycation than other sites. Lys-199 (Garlick and Mazer, 1983), Lys-276,281,378,439,545 (Arasteh *et al*., 2014; Rondeau and Bourdon, 2011; Miranda and Outeiro, 2010). There is a close relationship between change in the structure of HSA and related change in the accessibility of epsilon amino groups of lysine and arginine amino acids. The molecular weight ofhuman serum albumin is 66.7 kilodalton (kD). The single chain ofHSA has 585 amino acids. There are 17 disulfide bonds in a single chain of HSA. The structure ofhuman serum albumin is mainly composed of Alpha helix and fewer beta turns (Peters,1996; He and Carter,1992; Choi and Foster,2002). HSA has 24 arginine which is also involved in the formation ofAGEs (Peters,1996). Along with the transport function of HSA, there are many physiological processes related to it like, maintaining the pH of the blood. There are various binding sites in HSA, for fatty acids (Curry *et al*., 1998; Koyama *et al*.,1997), for small solutes (Peters,1996; Otagiri,2005), and for heterocyclic compounds (Peters,1996; Otagiri, 2005). The modifications occurring on specific residues of glycated protein need to be studied (Lapolla *et al*., 2005). Previous glycation studies were available for albumin (Iberg & Fluckiger, 1986). The HSA glycation needs to be studied because ofits abundance (Peters, 1996).

The information regarding specific glycated peptide can be obtained from human serum albumin (Zhang *et al*., 2011). The advanced glycation end products formation and related details can also be obtained (Ahmed and Thornalley, 2005; Ahmed *et al*., 2005). The overall modifications in glycated human serum albumin and location of glycation sites can be studied by matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Frolov and Hoffman,2008; Brancia *et al*., 2006; Barnaby *et al*., 2010; Barnaby *et al*., 2011; Wa *et al*., 2007; Barnaby *et al*., 2011; Lee *et al*., 2007; Anguizola *et al*.,2013; Thornalley *et al*., 200). Uses of Liquid Chromatography-Mass Spectrometry (LC-MS) are previously reported for characterization of proteins from purified protein samples (Aebersold and Mann, 2003; Yates *et al*., 2005; Mann and Jensen, 2003). The MS uses electrospray ionization to study macromolecules (Fenn *et al*., 1989).

The LC-MS gives accurate information about the changes in the molecular mass of amino acids. (Aebersold and Mann, 2003; Sadygov *et al*., 2004). Mass spectrometry provides detailed information about post-translation modifications occurring on peptides (Mann and Jensen, 2003; Jensen, 2004). The LC-MS analysis is coupled with software systems to provide a search against a given database of proteins (Sadygov *et al*., 2004; Fenyo and Beavis, 2002; Creasy and Cottrell, 2002). The specific sites on which glycation related post-translational modifications are occurring can be understood with mass spectrometry (Mann and Jensen, 2003). The facility of searching the database to understand the sequence of amino acids in a protein is done through the existing databases (Cantin and Yates 2004; Wu *et al*., 2003).

MATERIAL AND METHODOLOGY

Data collection from RCSB-PDB: The crystallographic structure of human serum albumin was obtained from the Protein Data Bank (PDB), a repository for the 3-D structural data of large biological molecules, such as proteins. The PDB ID for the crystallographic structure of human serum albumin was 1AO6. Ligand bound human serum albumin structures were also obtained from the RCSB protein data bank.

Data visualization by Swiss PDB Viewer: The file format initially used by the PDB was called the pdb file format. The structure files may be viewed using one ofseveral open-source computer programs such as Swiss PDB Viewer. To understand the 3D structures ofalbumin, we have used molecular graphics software for data visualization i.e. Deep swiss PDB viewer, which is an interactive program for viewing 3D structures of the protein. The color mend of the viewer was used, which shows a palette of 20 colors and provides information about the accessibility of amino acids. All downloaded files of the HSA structures were viewed one by one to study the accessibility oflysine and arginine residues.

Mass spectroscopy (LC-MS^E) analysis

Preparation of glycated HSA: The protein samples were prepared in double distilled water. The *in-vitro* glycation of proteins was performed by incubating equal volume (50µl) of protein with 1M glucose solution dissolved in 0.2M phosphate buffer, at 37°C for either 3, 6 or 10 days.

Trypsin digestion: 100 micrograms of glycated protein was used, which was solubilized in hundred microlitres of NH_4HCO_3 having a concentration of 50 mM. 0.1% Rapigest SF (Waters Corporation, MA, USA) was used to increase proteolytic cleavage. Then the sample was incubated in a boiling water bath at 80 °C. After that, 100 mM DDT was used for the reduction of protein at 50 °C in a boiling water bath for 15 minutes. The alkylation of reduced protein was carried out with 200 millimolar iodoacetamide and the samples were incubated for 20 minutes in dark. The digestion ofprotein was carried out overnight at 37 °C with a 10:1 ratio oftrypsin. The reaction was stopped by adding 2 microlitres offormic acid.

LC-MS Analysis: With the concentration of hundred nanograms per microlitre, digested peptides were used for analysis (cheng *et al*.,2009), using nano ACQUITY UPLC online coupled to SYNAPT HDMS system (MS^E) (Waters Corporation, Milford, USA). The Protein Lynx Global Survey software (PLGS version 2.4, Waters Corporation, India) was used for the analysis ofdata obtained from MS^E analysis. The UniProt human database was utilized for data searching in PLGS.

RESULT AND DISCUSSION

Screening of HSA- ligand complexes

Study of the structure of human serum albumin crystallographic structure: To study possible conformations of human serum albumin we have used crystal structure of HSA having PDB ID: 1AO6 as a standard structure.

Study of HSA-Ligand crystallographic structures: Ligand is a substance that forms a complex with a biomolecule. Such ligand-bound human serum albumin structures were also obtained from the protein data bank. In order to get a holistic overview of the structure and confomation of human serum albumin and related change in the accessibility of amino acids, other structures of HSA ligand - complex containing different conformations of human serum albumin were accessed from Research Collaboratory for Structural Bioinformatics - Protein Data Bank. The structural changes after binding of a particular ligand were studied, following ligands and identification codes were used for this study:

LIGAND ID's: 1FL, 4EB, 9DN, 9DS, 9NE, 9NF, 9NR, 9NV, ACD, ALY, AZQ, AZZ, B3I, BAB, BAH, BAI, BAM, BLA, C1F, CA, CIT, CL, DAO, DIO, DKA, DZP, ESI, FUA, GOL, HEM, HLT, IBP, IDB, MN, IMX, IO3, IOS, IPX, IQX, LI8, LPX, LZQ, MCL, MYR, NA, NPS, OLA, OPB, P1Z, P28, PFL, PJZ, PLM, PO4, RWF, SAL, SEP, SO4, STE, SWF, T33, T44, T4A, TYS, ZN.

The identification codes: For each ligand-bound human serum albumin there is particular structural ID.

2BXE, 1YSX, 2XVU, 2XVV, 2XVQ, 2XSI, 2XWO, 2XVW, 2XW1, 1GNJ, 2I2Z, 2BX8, 2BXI, 2BXK, 3B9L, 3B9M, 1BKE, 2VUE, 2BXA, 1TFO, 1E7F, 2BXL, 1E7E, 2VDB, 2BXF, 2VUF, 1N5U, 109X, 1E7B, 1E7C, 2BXG, 2BXN, 2BXK, 2BXM, 2BXQ, 3LU6, 2YDF, 2BXH, 3LU7, 3LU8, 3CX9, 3JQZ, 3A73, 3SQJ, 1HK4, 1HK5, 1H9Z, 1BJ5, 1GNI, 2BXB, 2BXC, 1E7A, 1E7H, 2BXD, 3JRY, 1E7I, 1HK1, 1HK2, 1HK3, 1E7G, 1HA2, 2BXO, 2BXP, 2I3O

Data visualization by Swiss-PDB Viewer: The accessible amino acid residues which are present in protein were studied by checking the surface accessibility of amino acids in Swiss PDB Viewer with help of 3D structures accessed from RCSB-PDB.

All downloaded files of HSA-ligand complex structures were studied, in addition to crystal structure of human serum albumin to check the accessibility of lysine and arginine residues. The color mend of the Swiss PDB Viewer was used, which shows an orderly palette of 20 colors, which provides information about accessibility of amino acids.

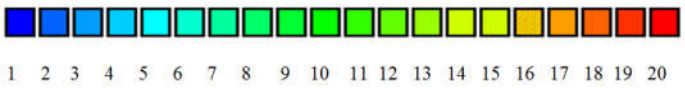

Figure 1. Orderly palette of 20 colors (providing 5% increments on a 0 to 1 scale)

Dark violet color denotes fully buried amino acid and red color is allotted to amino acids showing a minimum 75% surface accessibility. Generally, amino acids having more than 25% of accessibility can undergo glycation. So, 20 colors as shown in figure 4.2.1. provides accessibility information by 5% increments from the first violet color to the last red color with increment on 0 to 1 scale. That's why the first five colors i.e. 1,2,3,4,5 means amino acid has 0 to 25% accessibility and is buried, so not involved in glycation. The remaining 15 colors from 6 to 20, have accessibility of 30 to 100 % and are involved in glycation.

Data visualization: Molecular Graphics Software i.e. DeepView –Swiss-PdbViewer (Version 4.04) was used for this study.

Viewing the data: The file format initially used by the proein data bank was called as the pdb file format. These structure files may be viewed using one of several open-source computer programs such as the Swiss PDB viewer. We have studied the crystal structure of human serum albumin as the standard structure and screened for lysine and arginine residues showing more than 25% accessibility.

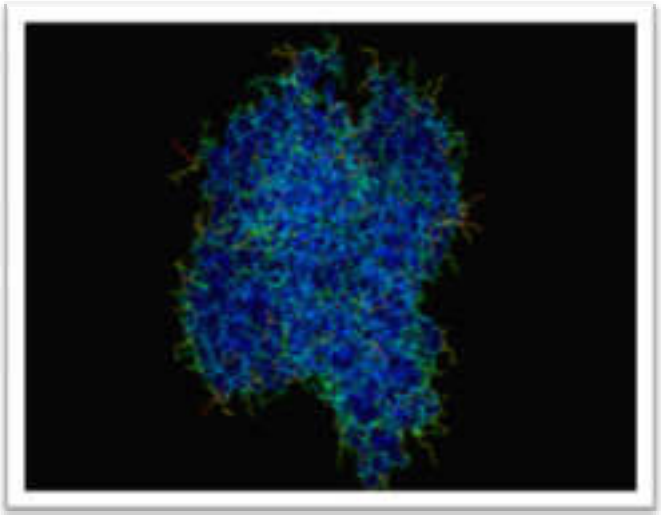

Figure 2. Structure of HSA with accessibility colors (using Swiss-PDB Viewer version 4.1)

Afterward, we have studied structures of human serum albumin – ligand complexes, to understand structural variations of human serum albumin and related change in the accessibility of lysine and arginine amino acids. After analyzing all 3D structures, we have screened lysine and arginine residues, which are always accessible for sugar molecules under any confirmation of human serum albumin.

Identification of highly accessible Lysine & arginine

Role of accessible lysine and arginine residues in glycation: Glycation of epsilon amino group of lysine and arginine is known previously (Zhao et al.,1997). Due to the presence of a greater number of lysine, HSA is highly glycated in presence of sugar molecules (Garlick and Mazer,1983; Iberg and Fluckiger,1986). Although there are 59 lysine residues, only a few of them are highly glycated (Zhang et al, 2008a). So, all lysine's are not glycated at the same rate, there are few lysine residues which glycate more as compared to other lysine's.

Sequence analysis of human serum albumin:

```
MKWVTFISLLFLFSSAYSRGVFRRDAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPF
EDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEP
ERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLF
FAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAV
ARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLK
ECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYAR
RHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFE
QLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVV
LNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTL
SEKERQIKKQTALVELVKHKPKATKEQLKAVMDDFAAFVEKCCKADDKETCFAEEGKKLV
AASQAALGL
```

Figure 3. 609 amino acid sequence of human serum albumin with 1 letter codes

In sequence of human serum albumin, first 24 amino acids are for signal sequences and these pro-peptide portions are not observed in the transcribed, translated, and transported protein but present in the gene. There are 609 amino acids in this sequence with only 585 amino acids in the final product are observed in the blood.

The positions of lysine and arginine amino acids in human serum albumin: There are two chains of human serum albumin, chain A and chain B. Both the chains are identical in structure and sequence of amino acids.

Table 1. Positions of A) Lysine B) Arginine on human serum albumin

A)	4	162	274	402	525	B)	10	222
	12	174	276	413	534		81	257
	20	181	281	414	536		98	336
	41	190	286	432	538		114	337
	51	195	313	436	541		117	348
	64	199	317	439	545		144	410
	73	205	323	444	557		145	428
	93	212	351	466	560		160	445
	106	225	359	475	564		186	472
	136	233	372	500	573		197	484
	137	240	378	519	574		209	485
	159	262	389	524			218	521

Total no. of lysine's = 59 (for 585 amino acid sequence)
Total no. of lysine's = 24 (for 585 amino acid sequence)

The identified positions of accessible lysine and arginine amino acids in human serum albumin: After visualization of 1AO6 i.e. crystal structure of human serum albumin, in Swiss PDB viewer. We have observed that although there are 59 lysine residues, they are not equally accessible for sugar molecules. 48 lysine residues show the accessibility of more than 25%. Similarly, out of 24 arginine amino acids in human serum albumin, only 6 residues of arginine have the accessibility of more than 25%. It means remaining 11 lysine residues and 18 arginine residues are buried inside the structure and have less than 25% accessibility. The following table shows the identified positions of accessible lysine and arginine residue having more than 25% accessibility for sugar molecules.

Table 2. Positions of A) Lysine B) Arginine having >25% accessibility on crystal structure of human serum albumin

A)	12	174	276	402	538	B)	81
	20	181	281	432	541		114
	41	190	313	436	545		117
	51	195	317	439	557		186
	64	205	323	444	560		209
	93	212	351	466	564		410
	136	225	359	475	573		
	137	233	372	500	574		
	159	240	378	519			
	162	262	389	524			

Accessible lysine's on human serum albumin = 48
Accessible arginine's on human serum albumin = 6

Positions of surface accessible lysine amino acids in crystal structure of human serum albumin which are more prone to glycation:

Positions of lysine residues according to the 585 amino acid sequence: 12, 20, 41, 51, 64, 93, 136, 137, 159, 162, 174, 181, 190, 195, 205, 212, 225, 233, 240, 262, 276, 281, 313, 317, 323, 351, 359, 372, 378, 389, 402, 432, 436, 439, 444, 466, 475, 500, 519, 524, 538, 541, 545, 557, 560, 564, 573, 574.

Positions of lysine residues according to the 609 amino acid sequence: 36, 44,65, 75, 88, 117, 160, 161, 183, 186, 198, 205, 214, 219, 229, 236, 249, 257, 264, 286, 300, 305, 337, 341, 347, 375, 383, 396, 402, 413, 426, 456, 460, 463, 468, 490, 499, 524, 543, 548, 562, 565, 569, 581, 584, 588, 597, 598.

Positions of surface accessible arginine amino acids in crystal structure of human serum albumin which are more prone to glycation

Positions of arginine residues according to the 585 amino acid sequence: 81, 114, 117, 186, 209, 410

Positions of arginine residues according to the 609 amino acid sequence: 105, 138, 141,210, 233, 434.

As per our observation 48 lysine residues and 6 arginine residues show accessibility of more than 25%. But when a particular ligand binds with human serum albumin, the confirmation of human serum albumin changes significantly. The change in the structure of the protein after drug interaction is due to the binding sites and binding affinity of that drug. Although there are two main binding sites present in human serum albumin. The interaction of ligand molecules with protein is not limited to these binding sites. Different ligands bind with serum albumin in various locations. Many times, even a large number of small molecules such as glucose can alter the structure of human serum albumin due to the process of glycation. As we know glucose reacts with accessible amino acids, it is important to understand the positions of lysine and arginine on HSA, which are always accessible even after the binding of different molecules/drugs. To identify such amino acids our approach is to screen positions of amino acids which are on the surface and are accessible on the crystal structure of HSA, but when the ligand binds amino to HSA, acid residues to get buried inside the structure and becomes inaccessible for small molecules for binding. So, in such case buried amino acid positions of lysine and arginine residues are removed from screened 48 positions of lysine and 6 positions of arginine.

Identified positions of highly accessible lysine and arginine residues after study of different confirmations of human serum albumin: After visualization and analysis of HSA-ligand complexes, we have screened 19 positions of lysine and 3 positions of arginine, which are always accessible for interaction even after binding with various ligands. If, after binding of particular ligand surface accessible lysine / arginine is getting buried inside structure and so becomes inaccessible for interaction with sugar molecules, then we removed those positions to get highly accessible amino acids. Hence, these identified sites are more prone to glycation due to their high accessibility as shown in figure 4.

Highly accessible lysine's on human serum albumin = 19.
Highly accessible arginine's on human serum albumin = 3

Positions of highly surface accessible lysine amino acids in every conformation of human serum albumin which are more prone to glycation

Positions of lysine residues according to the 585 amino acid sequence: 12, 51, 205, 225, 240, 262, 276, 313, 317, 359, 372, 378, 439, 444, 519, 538, 541, 560, 573.

Positions of lysine residues according to the 609 amino acid sequence: 36, 75, 229, 249, 264, 286, 300, 337, 341, 383, 396, 402, 463, 468, 543, 562, 565, 584, 597.

Positions of highly surface accessible arginine amino acids in in every conformation of human serum albumin which are more prone to glycation

Positions of arginine residues according to the 585 amino acid sequence: 81, 114, 209

Positions of arginine residues according to the 609 amino acid sequence: 105, 138, 233

A) B)

12	174	276	462	538
20	181	281	492	541
41	190	313	496	545
51	195	317	439	557
64	205	323	444	560
93	212	351	466	564
136	225	359	476	573
137	232	372	500	574
150	140	378	519	
162	262	388	524	

81
114
117
186
209
410

Figure 4. Positions of (A) Lysine (B) Arginine having >25% accessibility on the all studied HSA-Ligand complex structures of human serum albumin

Analysis of post translation modifications on human serum albumin by LC-MS[E]: Various methods were reported to determine glycated albumin such as colorimetric, enzymatic, chromatographic, electrochemical, immunochemical but nowadays mass spectrometry is the first choice for researchers to study post-translational modification analysis.

Mass spectrometry can separate molecules on the basis of mass to charge ratio. That's why it is helpful in analyzing the location and type of post-translational modifications (Bai *et al.,* 2012).

Glucose was used to study glycated albumin and glycation products in the form of advanced glycation end products were studied by liquid chromatography-mass spectroscopy (LC-MS).

Our identified positions of surface accessible amino acids (lysine) which are more prone to glycation:

Positions according to the 585 amino acid sequence: 12, 51,205, 225, 240, 262, 276, 313, 317, 359, 372, 378, 439, 444, 519, 538, 541, 560, 573

Positions according to the 609 amino acid sequence: 36, 75, 229, 249, 264, 286, 300, 337, 341, 383, 396, 402, 463, 468, 543, 562, 565, 584, 597

Our identified positions of surface accessible amino acids (arginine) which are more prone to glycation:

Positions according to the 585 amino acid sequence: 81, 114, 209

Positions according to the 609 amino acid sequence: 105, 138, 233

As in glycation reaction, different glycation adducts are formed. The LC-MS spectra of in vitro glycated sample shows that sample was properly glycated as shown in Figure 5.

The glycation and resulting AGE's are responsible for the formation of various glycation adducts on Lysine and Arginine amino acids as given in Table 3.

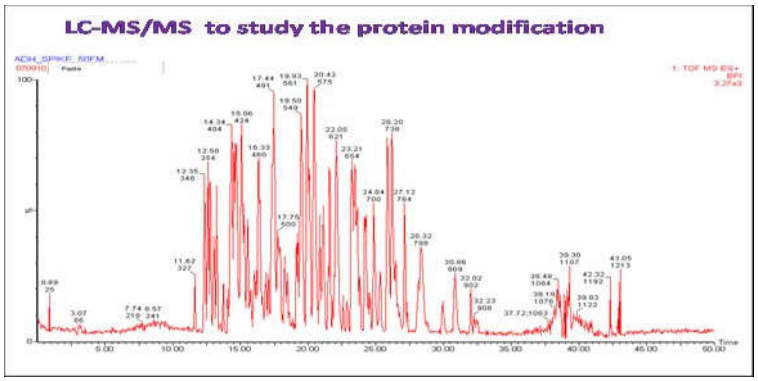

Figure 5. LC-MS spectra of glycated HSA for protein modification

Table 3. Glycation products involving lysine and arginine residues

Sr. No.	Amino acid involved	Glycation adduct	Δ M (Da)	Abbreviation
1	Lysine	Fructosyl-lysine	162.0528	FL
2	Lysine	Fructosyl-lysine-1H2O	144.0423	FL-1H2O
3	Lysine	Fructosyl-lysine-2H2O	126.0317	FL-2H2O
4	Lysine	Nε-Carboxyethyl-lysine	72.0211	CEL
5	Lysine	Nε-Carboxymethyl-lysine	58.0055	CML
6	Lysine	Pyrraline	108.0211	Pyr
7	Arginine	Nε-(5-(2,3,4-Trihydroxybutyl)-5-hydro-4-imidazolon-2-yl)ornithine	144.0423	3-DG-H1
8	Arginine	Tetrahydropyrimidine	144.0423	THP
9	Arginine	Imidazolone B	142.0266	IB
10	Arginine	Argpyrimidine	80.0262	ArgP
11	Arginine	Nε-(5-Hydro-5-methyl-4-imidazolon-2-yl)ornithine	54.0106	MG-H1
12	Arginine	Nε-(5-Hydro-4-imidazolon-2-yl)ornithine G-H1	39.9949	G-H1
13	Lysine / Arginine	1-Alkyl-2-formyl-3,4-glycosyl-pyrrole	270.0740	AFGP

The analysis by LC-MS provided information about the increase in molecular mass of glycated peptides as compared to un-glycated peptides.

The type of modification in the form of glycation adducts were observed on the basis of change in molecular mass of peptide as shown in Figure 6 and Figure 7.

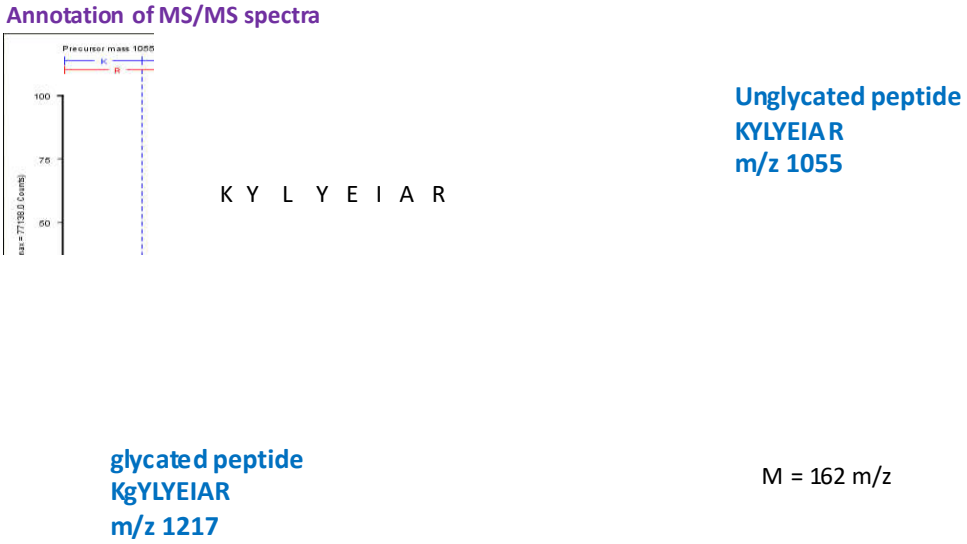

Figure 6. Glycation adducts involving lysine Fructosyl-lysine FL ΔM 162.0528 Da

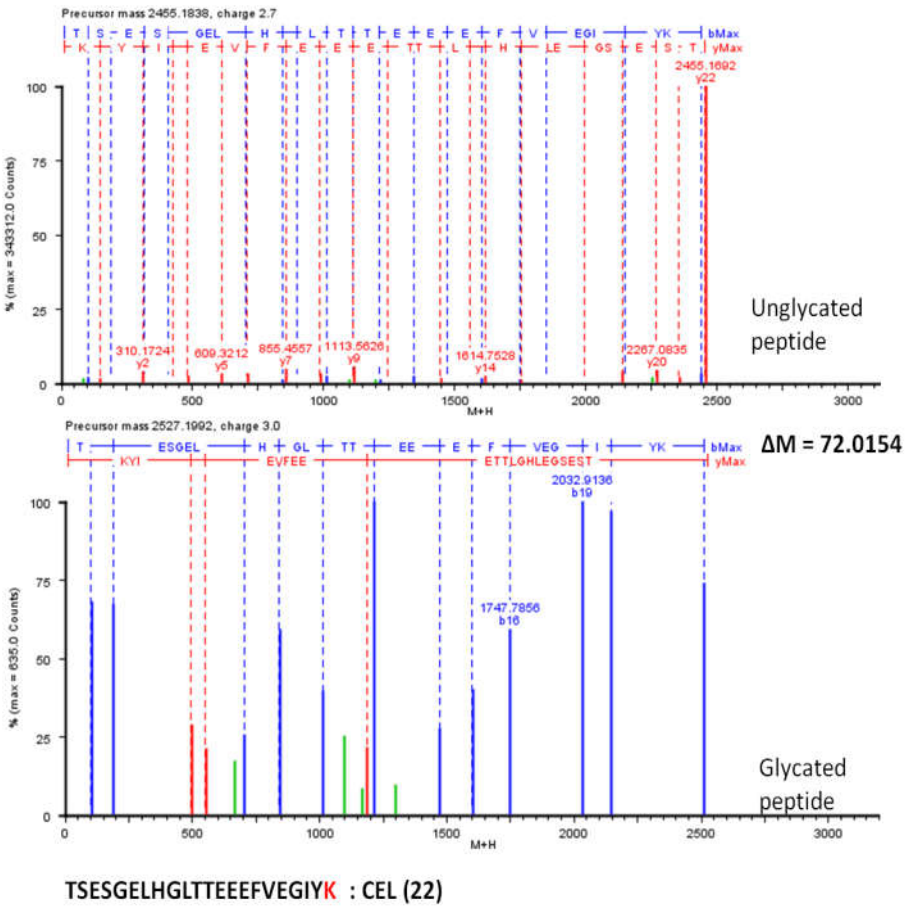

Figure 7. A representative MS/MS annotation of glycation modification of Carboxyethyllysine (CEL) showing increase in mass of 72.0154 Da

The data obtained from glycated HSA with help of LC-MS:

Table 4. PTM's of lysine's at known specific positions which are more prone to glycation due to higher accessibility on glycated HAS

Sr. No.	Positions on HSA sequence (according to 609 amino acid sequence)	Positions on HSA sequence (according to 585 amino acid sequence)	Post translation modifications in the form of glycation adducts identified by LC-MS[E]
1	36	12	KR-glycation, Pyrraline, Immidazolone A, Immidazolone B, Crossline, Pentosidine
2	75	51	Pyrraline, Pentosidine, CML
3	229	205	KR-glycation, Pyrraline, Immidazolone A, Immidazolone B, APGP
4	249	225	Crossline, Immidazolone A, Pentosidine
5	264	240	Crossline, APGP
6	286	262	KR-glycation,
7	300	276	Pyrraline, Crossline, Immidazolone B, CML
8	337	313	Pyrraline,
9	341	317	Immidazolone A
10	383	359	Immidazolone B
11	396	372	KR-glycation
12	402	378	CML, Immidazolone A, Immidazolone B
13	463	439	Crossline, Immidazolone A
14	468	444	Pentosidine
15	543	519	Crossline, Immidazolone B,
16	562	538	APGP
17	565	541	KR-glycation, APGP
18	584	560	CML, Immidazolone B,
19	597	573	Immidazolone A

Table 5. PTM's of arginine's at known specific positions which are more prone to glycation on glycated HAS

Sr. No.	Positions on HSA sequence (according to 609 amino acid sequence)	Positions on HSA sequence (according to 585 amino acid sequence)	Post translation modifications
1	105	81	KR-glycation, Immidazolone A
2	138	114	Pyrraline, Immidazolone A
3	233	209	CML, Immidazolone B, Immidazolone A

The accessibility of amino acid and protein structure was determined by glycation pattern (Menella et al.,2006; Kueper et al.,2007). Crystallographic structure Human serum albumin and HSA-ligand complex structures were studied and the positions of amino acids were identified, which were always accessible for sugar molecules even after binding of different ligands. Numbers of lysine which are prone to glycation were more while arginine are less, so lysine residues were mainly involved in glycation. The in-vitro glycated samples of HSA were analysed by liquid chromatography-mass spectrometry and at our identified positions number of additional post translation modifications at were observed as given in Table 4 and 5.

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