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Research Article

PSEUDOAFFINITY CHROMATOGRAPHY ENRICHMENT OF GLYCATED PEPTIDES FOR MONITORING ADVANCED GLYCATION END PRODUCTS (AGES) IN METABOLIC DISORDERS

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Abstract: Advanced Glycation End (AGE) products are produced due to diabetic progression and they are responsible for many complications in the diabetic disorder. The diabetic progression is measured, particularly following glycated hemoglobin using specific antibodies. However, the most abundant protein in blood, human serum albumin, is also found to be glycated which has a much shorter half life and gives information on short term glycemic control. In addition, glycated albumins are considered as markers of diabetic complications such as nephropathy, peripheral vascular calcification and also in Alzheimer's disease.

The glycation proceeds from the interaction between aldehyde group of sugar and the free amino group of the protein, resulting in the formation of Schiff's base, which undergoes a series of modifications leading to generation of imidazoyl derivatives of amino acids known as Amadori rearrangement products. The imidazoyl derivatives from arginine and lysine are the most prominent modifications observed in proteins in the presence of reducing sugar and these imidazoyl derivatives have an affinity towards certain transition metal ions. Based on our earlier exhaustive work on trapping the histidine peptides using transition metal ion, Cu(II) linked to imino-diacetate complex, we explored Cu(II) immobilized metal affinity chromatography (IMAC) as a potential tool for specific detection of glycated peptides of human serum albumin. Our results clearly demonstrate that Cu(II) IMAC is able to detect glycated peptides very efficiently while the non-glycated forms were not retained on the Cu (II) column as confirmed by LC-MS/MS analysis. We further discuss the utility of IMAC technology to enrich the detection of AGE products in plasma. We anticipate that these studies may provide valuable information on understanding disease pathologies and the potential of AGE products as biomarkers of various diseases including neurodegenerative, renal and cardiovascular diseases.

Keywords: Pseudoaffinity chromatography; Glycated peptides; Glycation end products; AGE; Metabolic disorders

Introduction

Diabetes is a state of metabolic disorder which is characterized by increased concentrations of blood sugar, either attributed to insufficient insulin production by the pancreas (type I diabetes) or due to the resistance to insulin (type II diabetes). The incidence of diabetes is increasing at an alarming rate and it is expected to reach to about 300 million by the year 2025 (King *et al.*, 1998). Numerous experimental evidences have emerged over the past couple of decades that indicate a correlation between increased blood glucose levels with increased risk of secondary complications in diabetes such as vascular, renal and retinal complications (Genuth *et al.*, 2005; Goh and Cooper, 2008) as well as in the development of atherosclerosis and age related neurodegenerative diseases like the Alzheimer's and Parkinson's disease (Vicente and Outeiro, 2010; Ko *et al.*, 2010; Lee *et al.*, 2009). Many of these secondary complications associated with

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hyperglycemia have been linked to the biochemical process of advanced glycation of proteins resulting in the Advanced Glycation End products (AGEs).

Unlike glycosylation which is enzymatically controlled process, glycation is an inevitable, non-enzymatic reaction between the reducing sugars and proteins occurring in all living system. The glycation process was first observed by Louis-Camille Millard in the year 1911 from the browning reaction when amino acid was incubated with D-glucose, the process which is now known as Millard reaction (Millard, 1912). Glycation is generally observed with proteins which are in relatively higher concentrations and which are long-lived as the process is substrate concentration dependent and due to its slow kinetics. It is a highly dynamic process that starts with the interaction between the aldehyde or ketone group of sugar and the free amino group of the protein resulting in the formation of Schiff's base, which undergoes series of reactions leading to the formation of heterogenous AGE products (Thornalley *et al.*, 1999).

The measurement of glycation can be used to determine an individual's long, medium and short term glycemia, as the glycation level of a protein with well known half-life is directly related to the glucose concentration to which it is exposed. For example, measuring the glycation of hemoglobin reflects blood glucose concentrations over a period of 6-8 weeks, while glycated albumin and ApoB can be used to estimate the glycemic index of previous 2-3 weeks and one week respectively. The rate of glycation of protein is increased in diabetic patients as compared to the normal subjects. One of the main clinical parameter apart from the measurement of blood glucose (SMBG) that is used to monitor the diabetes is the measurement of glycated hemoglobin (HbA_{1c}) of the red blood cell. The HbA_{1c} is generated by the addition of glucose to the α-amino group of the valine present at the terminal end of the hemoglobin β-chain (Shapiro *et al.*,1980) and many methods like cation exchange chromatography, affinity chromatography, immunoassay and isoelectric focusing methods have been developed to evaluate HbA_{1c} (Goldstein *et al.*, 1986). Owing to the long term half-life of the RBCs (120 days) the measurement of HbA_{1c} levels serves as long term glycemic indicator over a period of 2 months. Although the joint guidelines issued by ADA and WHO in the 2009 has proposed the measurement of glycated hemoglobin as diagnostic criterion for diabetes (ADA, 2009; WHO, 2011), several studies have reported discrepancies in the HbA_{1c} as relevant indicator for glycemic index in patients with diabetes-associated pathologies such as hemolytic or renal anemia etc in which HbA_{1c} gives incorrect values and known to be unsuitable as marker of glycemic control (Bry *et al.*, 2001; Jeffcoate, 2004). A number of studies have reported measurement of glycated albumin which has a shorter half-life of 21 days as an alternative marker to HbA_{1c} for evaluating the glycemic control. Due to the shorter half-life and faster kinetics of *in vivo* glycation as compared to hemoglobin, measurement of glycated albumin could serve as a better marker of short-term glycemic index in several diabetes-associated pathologies (Garlick and Mazer, 1983; Iberg and Fluckiger, 1986). Numerous studies have reported the involvement of glycated albumin in the development of numerous diabetes associated complications such as nephropathy (Gugliucci, 2000), retinopathy (Okumura *et al.*, 2007) and Alzheimer's disease (Shuvaev *et al.*, 2001). Further the role of glycated albumin in peripheral vascular calcification (Yamada *et al.*, 2008) and arterial stiffening (Kumeda *et al.*, 2008) support the utility of glycated albumin as a marker in the detection of short term changes in glycemic index for diabetes and its associated pathologies.

Various methods have been used to characterize and quantify glycated proteins, including *in vitro* or *in vivo* glycated albumin. These include colorimetric methods such as thiobarbituric acid (TBA) (Elder and Kennedy, 1983) and nitro blue tetrazolium (NBT) (Mashiba *et al.*, 1992); enzymatic method (Kouzuma *et al.*, 2004); electrophoretic approach using phenyl boronate incorporated acrylamide gel electrophoresis (Morais *et al.*, 2009) and immunoassay method called enzyme–linked boronate immunoassay [ELIBA] (Ikeda *et al.*, 1998). Apart from these, separation-based assays that employ the use of ion-exchange chromatography and boronate affinity

chromatography (BAC) have been widely employed for separation and enrichment of glycated proteins and peptides prior to MS analysis (Zhang *et al.*, 2008).

In general, the glycation happens at amino groups as they are expected to be more reactive due to their greater nucleophilicity (Bunn *et al.*, 1979). Although the proteins contain many surface amino groups, it has been observed that only a few sites are preferentially glycated. This preferential glycation was further explained by the observation that those amino groups that were either closer to an imidazole moiety or part of lysine doublets were more susceptible to glycation (Iberg and Fluckiger, 1986; Acosta *et al.*, 2000). This was explained as the consequence of localized acid-base catalysis of aldimine/ ketoamine tautomerization (Watkins *et al.*, 1987).

Owing to the significant role played by histidine in the process of glycation and the affinity of few of the reported AGEs towards metal ions, in this work we have evaluated IMAC as a potential tool for studying the sites of glycation via capturing of histidine peptides and also study the affinity of glycated peptides to Cu(II) using glycated human serum albumin (HSA) as the model protein. In IMAC, the imidazole group of histidine is involved in the coordinate bond formation with the chelated metal ion (Porath, 1992; Sulkowski, 1989). Some of the peptides containing glycation modifications such as MG-H1, G-H1 and imidazolone B have an imidazole like group of histidine (Fig. 1) and can possibly contribute to the retention of glycated peptides on IMAC column.

Methodology

Chemical

Reagents ammonium bicarbonate, dithiothreitol, iodoacetamide, guanidine hydrochloride, human serum albumin, LC MS grade acetonitrile were purchased from Sigma, USA. All other used were of analytical grade and Milli-Q water was used for the experiments.

Trypsin digestion

The non-glycated and glycated HSA samples were dissolved in 100mM ammonium bicarbonate pH 8.5 buffer (AMBIC) containing 6M guanidine hydrochloride (GnHCL) to denature the protein followed by 15µL of 1M dithiothreitol (DTT) dissolved in 1M AMBIC (pH 8.5). The mixture was incubated at 60°C for 90min to reduce the protein. Reduced protein was carbamidomethylated by adding 36µL of 1M

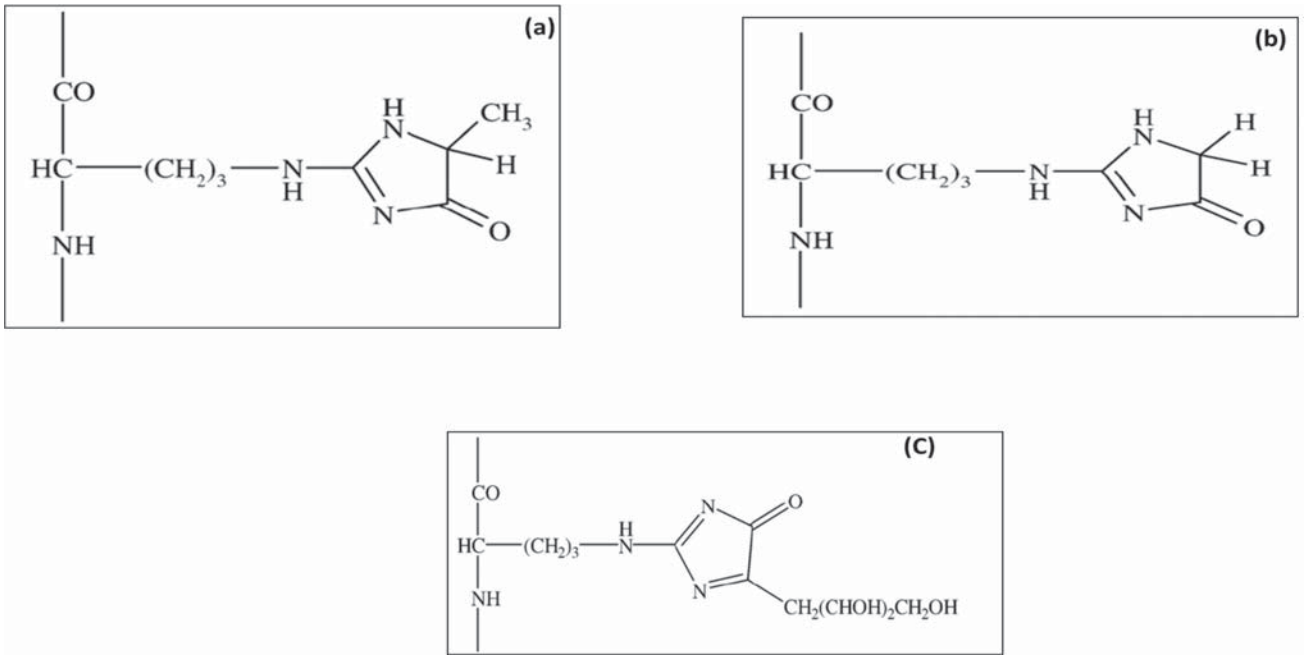

Figure 1: **Advanced glycation end products involving arginine (R) residue and having imidazole like group : (a) MG-H1 (ΔM = 54.0106 Da); (b) G-H1 (ΔM = 39.9949 Da); (c) Imidazolone B (ΔM = 142.0266 Da);**

iodoacetamide (IAA) dissolved in 1M NaOH. The mixture was then incubated for 45min at room temperature in dark. Guanidine hydrochloride and excess of DTT and IAA were removed using Biorad 10DG desalting column.

Cu(II)-IMAC selection

Cu(II)-IMAC selection was performed on AKTA FPLC (GE healthcare). CIM-IDA disk (12mm x 3mm) was used for selection of histidine containing peptides. The disk was first washed with 10 Column Volumes (CV) of milli Q water followed by 10 CVs of 50mM $CuSO_4$. Excess and loosely bound copper ions were removed by washing the disk with 10 CVs of elution buffer (25mM glycine, 0.5M NaCl, pH 3.0) followed by equilibration with 10 CVs of binding buffer (25mM MMA (MES, MOPS, sodium acetate), 0.5M NaCl, pH 7.0). The flow rate used was 2ml/min and the UV absorbance was monitored at 254nm. 0.5ml of tryptic digest was mixed with 0.5 ml of binding buffer and loaded into the CIM-IDA-Cu(II) monolithic column. After the sample was loaded the column was washed with binding buffer until the UV absorbance reached baseline. The bound peptides were then eluted with elution buffer in a single step pH elution. The eluted peptides were concentrated using speed vac before taking for MS analysis.

LC-MS/MS analysis

All the samples were analyzed on 6540 ESI-Q-ToF (Agilent Technologies, USA). The chromatographic separation was carried out on 1290 Infinity HPLC system (Agilent Technologies, USA) consisting of a binary pump, autosampler, thermostated column chamber, diode array detector (DAD) using 2.1 x 50mm, 1.8µm Zorbax C18 column the outlet of which was coupled to the ESI-Q-ToF and the data acquisition was performed using Mass Hunter® software.

The samples were ionized using Agilent jet streak electrospray ionization (AJS ESI) in the positive ionization mode with the following settings: gas temperature 350°C; drying gas at 6 l/min; nebulizer 45psi and capillary voltage of 3500V. The samples were analyzed on the auto MS/MS mode and centroid data storage. For data centroiding the following thresholds were used: for MS absolute threshold of 100 and relative of 0.01% while absolute of 5 and relative of 0.01% were used for MS/MS. Acquisition ranges were 100-2000m/z for MS, with scan rate of 2 spectras/s, and between 50-3000 m/z for MS/MS spectra (4 spectras/s). Data dependent analysis was performed and four precursor ions were selected per cycle, with active exclusion. Charge state preference was set for selection of peptides with 2 or more charges. Internal reference mass standards were used for the operation. The data analysis was done using the Agilent's Bioconfirm® software by manually curating the HSA sequence to add the modifications. Some of the modifications analyzed are given in Table 1.

Results

A variety of analytical techniques have been employed to analyze protein glycation. Browning and AGE fluorescence are characteristic of the Millard's product. The simplest means of assessing the degree of the reaction is to measure its absorbance, reflectance of light in the visible region. An alternative approach is to measure the fluorescence which develops prior to color attributed to some of the AGEs such as pentosidine, crossline and imidazolones are fluorescent. The other methods employed for analysis and measurement of AGEs include ELISA based measurement of AGEs, use of boronate affinity chromatography for enrichment of glycated proteins/peptides and mass spectrometric based characterization of AGEs.

The detection and identification of the glycated proteins and their glycation sites is complicated by the fact that the degree of glycation varies from trace amounts in short lived proteins to about 20-30% in long-lived proteins. Although a number of mass spectrometer based proteomics methods have been employed for studying protein glycation, it has its own limitations attributed to the limited dynamic range and the suppression of ionization of low abundant peptides in the analysis of complex mixtures like plasma or serum. The detection and identification of glycated proteins and the degree of glycation is complicated by the fact that their concentrations are relatively lower than the non-glycated form. To overcome the limitation

Table 1
Some of the glycation modifications that were studied with mass increase (Äm (Da)) and the glycation residue (K/R)

Modification	*Abbreviation*	*Δm (Da)*	*AA involved*
Fructosyl-lysine	FL	162.0528	K
N∈-Carboxymethyl-lysine	CML	58.0055	K
N∈-Carboxyethyl-lysine	CEL	72.0211	K
Pyralline	Pyr	108.0211	K
N∈-[5-(2,3,4-Trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine	3-DG-H1	144.0423	R
Tetrahydropyrimidine	THP	144.0423	R
Imidazolone B	IMD	142.0266	R
Argpyrimidine	Arg-Pry	80.0262	R
N∈-(5-hydro-5-methyl-4-imidazolon-2-yl)ornithine	MG-H1	54.0106	R
N∈-(5-hydro-4-imidazolon-2-yl)ornithine	G-H1	39.9949	R

associated with ion-suppression boronate affinity chromatography (BAC) has been employed to enrich the glycated proteins/peptides prior to MS analysis. The BAC is based on the interaction between boronic acid and the *cis-diol*-containing carbohydrate (Zhang *et al.*, 2008). Although BAC has been employed for enrichment of glycated proteins/peptides, it also has some practical limitations as it retains all types of glycated and glycosylated species from the sample, which necessitates additional separation steps prior to MS analysis.

As has been described previously, glycation occurs preferably at the amino groups that resemble histidine (≈ 5Å) (Acosta *et al.*, 2000) lead us to study the potential of IMAC for studying protein-glycation via selective enrichment of histidine peptides. In IMAC the selection of histidine containing peptides is achieved by the co-ordinate bond formation between the histidine of the peptide and the metal ion immobilized on the support.

The potential of IMAC as a tool for studying glycation was investigated with the tryptic digest of glycated HSA (both from Sigma and *in vitro* glycated). The peptides retained on the Cu(II)-IMAC column were eluted at pH 3.0 and further analyzed by LC-MS/MS. A total of 15 and 10 peptides were identified from the LC-MS/MS analysis of the Cu(II)-IMAC retained fraction of sigma and in-house glycated HSA respectively.

Cu(II)-IMAC retained peptides from glycated HSA

Only 5 of the 15 peptides identified from the LC-MS/MS analysis of the IMAC retained fraction of glycated-HSA, were found to contain histidine, while the binding of other 10 non-histidine peptides could be attributed to the interaction of AGE with Cu(II) (Table 2). Out of the 5 retained histidine peptides, no AGE modification was identified in 3 peptides while the remaining two histidine-peptides (HPEAKR and RDAHK) were found to be glycated. The retention of these two glycated his-peptides could be attributed to the interaction of the AGE with the metal ion as these two peptides were not retained on the Cu(II)-IMAC column from the tryptic digest of non-glycated HSA which was used as the control. One of the two histidine peptides that were identified only in the retained fraction of glycated HSA – HPEAKR had N∈-carboxyethyl-lysine (CEL) and imidazolone in 468K and 469R while the other peptide RDAHK was found to have imidazolone modification in 242R. Further, the presence of one mis-cleavage in each of these peptides substantiates the glycation as it makes the protein more resistant to proteolysis. Although the peptides HPEAK and RDAHK were identified in the LC-MS/MS analysis of non-glycated HSA, they were not retained on the Cu(II)-IMAC column which would indicate that the retention of IMAC column could be attributed to the interaction of CEL or more probably the

Table 2
List of peptides identified by LC-MS/MS analysis from the IMAC retained and eluted fraction from commercial glycated-HSA (Sigma)

Sequence	*SeqLoc*	*Missed*	*Pred Mods*	*TgtSeq Mass*	*Diff (Bio, ppm)*	*Score (Bio)*	*RT*	*Vol*	*MS/MS Count*
FPKAEFAEVSKLVTDLTK	A(247-264)	2	2*CML(+58.00548);1*FL-H2O(+144.04226)A257A249A264	2282.157	9.83	8.95	12.228	2465731	3
AWAVAR	A(237-242)	0	1*THP(+144.04226)A242	816.413	2.31	29.64	10.22	646753	1
FK	A(35-36)	0	1*Pyrr(+108.02113)A36	401.1951	-4.63	24.78	11.282	204851	0
CCTESLVNR	A(500-508)	0	1*MGH-1(+54.010565)A508	1191.5012	5.35	17.48	9.507	980981	1
RDAHK	A(24-28)	1	1*IMD(+142.02661)A24	767.3562	8.11	11.29	11.353	727291	0
ERQIKK	A(544-549)	2	1*IMD(+142.02661);1*CEL(+72.02113);1*FL-2H2O(+126.031695)A545A549A548	1140.5663	-2.66	32.86	9.48	484239	2
TPVSDRVTK	A(491-499)	1	1*GH-1(+39.994915)A496	1041.5455	6.36	20.26	12.361	1960977	1
TPVSDRVTK	A(491-499)	1	1*Arg-Pyr(+80.026215); 1*CML(+58.00548)A496A499	1139.5823	-3.84	24.91	9.239	1000082	4
LKCASLQK	A(222-229)	1	1*FL-2H2O(+126.031695)A229	1072.5587	2.74	30.04	10.681	972671	1
LKCASLQKFGER	A(222-233)	2	1*CEL(+72.02113); 1*FL-2H2O(+126.031695); 1*GH-1(+39.994915)A223A229A233	1673.8083	-6.52	11.95	11.485	1437017	2
HPEAKR	A(464-469)	1	1*CEL(+72.02113);1*IMD(+142.02661) A468A469	950.4458	4.2	22.28	10.992	5013141	0
QRLKCASLQK	A(220-229)	2	1*Arg-Pyr(+80.026215);1*FL-H2O(+144.04226)A221A223	1454.7552	2.56	26.22	11.437	2820557	0
NECFLQHK	A(123-130)	0		1074.4917	3.5	57.28	9.044	1513297	1
SEVAHR	A(29-34)	0		697.3507	2.52	27.45	5.104	1443127	0
SLHTLFGDK	A(89-97)	0		1016.5291	3.43	66.57	10.219	10158613	2

imidazolone modification which is a closer analogue of the imidazole of histidine. As it can be seen from table 2, that apart from the reported Nõ-carboxymethyl-lysine (CML) which has an affinity for divalent metal ions other glycations also seem to exhibit some degree of affinity towards Cu(II) thereby resulting in the retention of these peptides in Cu(II)-IMAC column, while their non-glycated forms were not retained on the Cu(II)-IMAC column.

Further, it was also observed that IMAC capturing helped in the enrichment of some of the glycated peptides which were not identified in the LC-MS/MS analysis of the total tryptic digest of glycated HSA. This also further indicates that only some fraction of HSA in glycated and thereby detection of the glycated peptides becomes difficult due to the effect of ion suppression. Although the peptides with sequence AWAVAR and CCTESLVNR were identified in the LC-MS/MS analysis of the tryptic digest of glycated HSA (Sigma, USA), their glycated forms were only identified after IMAC capturing. Although the peptide AWAVAR was identified with THP modification at 242R, it is also possible that it could well be N∈-[5-(2,3,4-trihydroxybutyl)-5-hydro-4-imidazolon-2-yl]ornithine (3-DG-H1) glycation rather than tetrahydropyrimidine (THP) as both these modification will result in a mass difference of 144.0423Da only. The retention of this peptide on the Cu(II)-IMAC column could be attributed to the imidazole like group in 3-DG-H1. This could be partially due to the reason that the glycated forms of these peptides would have been in relatively lower concentrations and hence were detected only after IMAC enabled the enrichment of these peptides. Similarly the other glycated peptides ERQIKK and LKCASLQKFGER were identified only in the analysis of IMAC retained fraction. Also the peptide TPVSDRVTK with Nõ-(5-hydro-4-imidazolon-2-yl)ornithine (GH-1) modification was identified only after IMAC while the same peptide with Arg-Pyr and CML modification was identified in both the LC-MS/MS analysis of the total digest and the IMAC retained fraction.

Thus the identification of 5 additional peptides having different AGE modifications such as THP, MGH-1, imidazolone B (IMD), GH-1, CEL (Table 1) apart from the more prevalent CML indicate that most of the AGE products can interact with metal ion and it is not only restricted to CML. Further the use of IMAC as an enrichment tool enabled in the identification of additional glycated peptides, which could hold the key in understanding the mechanism and progression of glycation.

Cu(II)-IMAC retained peptides from glycated HSA (in-house)

In the experiment performed with in-house glycated HSA, which was produced by incubation of HSA with glucose, similar results were observed wherein some of the glycated peptides were identified only after the IMAC capture step. The LC-MS/MS analysis of the IMAC retained and eluted fraction resulted in the identification of seven glycated and 3 non-glycated peptides (Table 3). All the three non-glycated peptides were histidine containing peptides that were commonly identified from the LC-MS/MS analysis of Cu(II)-IMAC captured fraction from both non-glycated HSA (control) and the sigma glycated HSA.

Similar to the results observed with glycated-HSA (Sigma), most of the glycated peptides that were identified from the Cu(II)-IMAC fraction were identified only after IMAC and were not previously identified in the total digest. It was also observed that the three non-glycated histidine peptides were also not identified in the total digest and this could be attributed to the poor digestion efficiency because of the proteolytic resistance due to glycation. Among the glycated peptides, except the peptide LKECCEKPLLEK, all other peptides that are reported in table 2 were identified only after the Cu(II)-IMAC selection.

Comparison of the glycated peptides retained on Cu(II)-IMAC column from both the sigma and in-house glycated HSA samples, it could be seen that majority of the glycated peptides having identical glycation profiles were retained reproducibly, which further validates the affinity of different AGEs towards immobilized Cu(II).

Table 3
List of peptides identified by LC-MS/MS analysis from the IMAC retained and eluted fraction from *in-house* glycated-HSA

Sequence	*SeqLoc*	*Missed*	*Pred Mods*	*TgtSeq Mass*	*Diff (Bio, ppm)*	*Score (Bio)*	*RT*	*Vol*	*MS/MS Count*
CCTESLVNR	A(500-508)	0	1*MGH-1(+54.010565)A508	1191.5012	2.99	25.35	9.524	323797	1
ERQIKK	A(544-549)	2	1*IMD(+142.02661);1*CML (+58.00548);1*FL-2H2O (+126.031695)A545A548A549	1126.5506	-6.17	20.18	9.653	228915	1
HPEAKR	A(464-469)	1	1*CEL(+72.02113);1*IMD (+142.02661)A468A469	950.4458	2.19	27.67	11.022	340047	0
LAK	A(284-286)/ A(373-375)	0	1*CML(+58.00548)A375	388.2322	7.21	19.13	19.882	129797	0
LKECCEKPLLEK	A(298-310)	1	1*CML(+58.00548);2*FL (+162.052825)A300A310A305	1927.9006	7.28	19.53	11.601	1748297	4
QRLKCASLQK	A(220-229)	2	1*Arg-Pyr(+80.026215);1*FL-H2O (+144.04226)A221A223	1454.7552	2.56	26.21	11.466	427302	0
TPVSDRVTK	A(491-499)	1	1*GH-1(+39.994915)A496	1041.5455	5.67	22.67	12.376	402286	1
SEVAHR	A(29-34)	0		697.3507	2.08	28.25	5.102	854216	0
SLHTLFGDK	A(89-97)	0		1016.5291	3.5	62.38	10.278	382224	1
NECFLQHK	A(123-130)	0		1074.4917	2.86	53.16	9.073	278706	1

Conclusions and potential applications

Previous studies by Monnier and his co-workers have clearly indicated the affinity of only CML and CEL modified lysine residues to bind divalent metal ions like Cu(II) owing to its EDTA like configuration (Dwyer and Mellor, 1964) . The results of our study highlight the affinity of other glycation modifications like MGH-1, IMD, GH-1 which are usually associated with arginine and have been identified reproducibly from both in-house glycated and sigma glycated HSA. With the exception of few lysine and arginine glycations many of the sites of modification have been identified newly in this study. Among the Cu(II)-IMAC selected peptides, especially those associated with arginine, except 496R, most of the other sites are new to this study.

Although most of the glycation studies have focused primarily on the modifications involving lysine residues and the amino terminal, using this approach we were able to enrich and identify some of the glycation modification of arginine residue, which were otherwise not detected in the analysis of total digest. This is the basis for further improvement to detect many AGE products in the plasma.

This approach of Cu(II)-IMAC selection of peptides can also be used to study the progression of glycation over a period of time and to study the sites of modification correlating with the progress of glycation. Most of these modifications could be at or near the drug binding sites and understanding the glycation and its progress will help in designing new drugs.

These AGEs especially MGH-1, GH-1 and IMD which have imidazole like structures similar to that of histidine could potentially be involved in the increased Cu(II) binding by glycated proteins and subsequently result in diabetes associated pathologies.

Further, this Cu(II)-IMAC selection can also be investigated for its potential as a quantitative tool for estimating the glycemic index based on the retained and eluted glycated peptides.

Conflict of interest

Authors declare that there is no conflict of interest

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