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Received November 14, 2012
Revised January 14, 2013
Accepted January 18, 2013

Research Article

Monotopic modifications derived from in vitro glycation of albumin with ribose

Post-translational modifications are significant reactions that occur to proteins. One of these modifications is a non-enzymatic reaction between the oxo-group(s) of sugars and amino-group(s) of protein – glycation. This reaction plays an important role in the chronic complications of diabetes mellitus, or in the aging process of organisms, that is, it has an important role in the pathophysiology and "normal" physiology of animals. In the work presented here, we studied the glycation of albumins (HSA and BSA). Methodologically, we used nano-LC coupled to a QTOF mass spectrometer. In vitro-modified proteins were cleaved by trypsin and the arising peptides were separated on a C18 nano column with a trap-column. Peptides and their modifications were analysed with a high-resolution QTOF mass spectrometer with a mass determination precision of better than 5 ppm. Non-enzymatic in vitro reaction products between albumin and ribose were identified. Besides well-known carboxymethyl lysine, new modifications were determined – creating mass shifts of 78 and 218. The origin of the first modification is discussed and its possible structure is presented. In addition, a mass shift of 132 belonging to a Schiff base was also identified. The location of all the modifications within the structure of the proteins was determined and their reactivity to various oxo-compounds was also examined.

Keywords:
Advanced glycation end product (AGE) / Albumin / CE-MS / Glycation / LC-MS/MS
DOI 10.1002/elps.201300014

1 Introduction

In the non-enzymatic glycation of proteins, free amino groups on the peptides/proteins react with the carbonyl groups of reducing sugars without the catalytic action of enzymes. This reaction was first described by Louis Maillard [1], who observed the browning of protein when heated with sugars, and in his honour it is called the Maillard reaction. It initially appeared that this reaction is only relevant to food chemistry, but in 1971 it was found that glycation takes place in every living organism [2], especially if the concentration of sugar in the blood is increased. Non-enzymatic glycation takes place in three steps which can be designated initiation, propagation and termination, analogous to chain radical reactions.

In the first step, the so-called early glycation products are formed. This involves the condensation of reducing sugars (mainly glucose, but also ribose, fructose, pentose, galactose, mannose, ascorbate and xylulose) with the ε-amino group of a lysine residue (alternatively the amino group of a terminal arginine amino acid) and the formation of an unstable Schiff base, which rapidly rearranges to a much more stable ketoamine called an Amadori product [3]. The variables that regulate it in vivo are the concentration of reducing sugar and protein, the half-life of the protein, the free amino group reactivity and the permeability of cell walls for sugars. Under in vivo conditions, the Amadori product reaches equilibrium after approximately 15–20 days and it accumulates in both the long- and short-lived protein [4]. During the propagation step, the Amadori products are resolved to carbonyl compounds (glyoxal, methylglyoxal, 3-deoxyglucosone), which are highly reactive and can again react with the free amino groups of proteins, thus promoting a non-enzymatic glycation. In the last step, the above-mentioned propagators react with free amino groups and by oxidation, dehydration and cyclising reactions form advanced glycation end products (called AGEs). These products are thermodynamically stable and terminate the non-enzymatic glycation reaction [5].

The amount of AGEs in the body reflects the equilibrium between their formation and catabolism (degradation in the tissues, renal excretion). A high AGE content forms in diabetic patients and their excretion is insufficient in patients with renal failure. In both groups, therefore, there is an accumulation of AGEs in plasma, which leads to the production of toxic substances, interaction with the basal membrane and bonding to lipoproteins and collagen (a common long-lived protein). The pathological formation of cross-linkages caused

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Abbreviations: AGE, advanced glycation end product; **CML**, carboxymethyl lysine; **SPL**, scheduled precursor list

by AGEs leads to an increased stiffness of matrix proteins, limiting their functionality and increasing their resistance to proteolytic enzymes [6].

The previously reported monotopic AGEs found in glycated proteins can be divided into two categories based on the modified amino acid. The lysine-derived AGEs include N^{ε}-(carboxymethyl)-lysine (CML, ΔM: +58.005), N^{ε}-(carboxymethylhydroxy)-lysine (CMhL, ΔM: +71.985), N^{ε}-(carboxyethyl)-lysine (CEL, ΔM: +72.021), pyrraline (ΔM: +108.021) and 1-alkyl-2-formyl-3,4-glycosyl-pyrrole (AFGP, ΔM: +270.074) [7–11].

The arginine-derived AGEs include imidazolone A (ΔM: +144.042) and B (ΔM: +142.027), N^{δ}-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-L-ornithine (argpyrimidine, ΔM: +80.026), N^{δ}-(4-oxo-5-dihydroimidazol-2-yl)-L-ornithine or 1-(4-amino-4-carboxybutyl)-2-imino-5-oxo-imidazolidine (αNFC-1 or G-H1, ΔM: +39.995), N^{δ}-(5-methyl-4-oxo-5-hydroimidazol-2-yl)-L-ornithine or N^{δ}-(4-methyl-5-oxo-4-hydroimidazol-2-yl)-L-ornithine or 2-iminoimidazolidinone (βNFC-1 or MG-H1, ΔM: +54.011) and tetrahydropyrimidine (ΔM: +144.042) [11–15].

Amino acids containing free amino groups can also form bitopic AGEs – cross-links. Again, the amino acids responsible for the formation of these crosslinks are usually lysine and arginine. The most widely known cross-linked structure is pentosidine (an imidazo[4,5-b]pyridinium molecule comprised of ribose, a lysine and an arginine residue with ΔM: +57.984). Other reported lysine-arginine-derived crosslinks are glucosepane (ΔM: +108.021), GODIC (ΔM: +21.984) and MODIC (ΔM: +36.000) [16–19].

Lysine-lysine-derived crosslinks include for example crossline (ΔM: +252.063), GOLD (ΔM: +33.984), MOLD (ΔM: +48.000), DOLD (ΔM: +138.032), vesperlysine A (ΔM: +99.995), vesperlysine B and C (ΔM: +114.011) and GLUCOLD (ΔM: +154.027) [20–28].

Serum albumin is produced in hepatocytes and is the most abundant protein in blood plasma (constituting 55–65% of all plasma proteins). However, the albumin in plasma is only approximately 42% of the albumin in the body, the rest can be found in the tissues (mainly subcutaneous tissue and muscles) and a small amount penetrates the blood–brain barrier into the cerebrospinal fluid. Albumin is a soluble protein with an ellipsoidal shape (in contrast to other plasma proteins, which are glycoproteins). It consists of a single polypeptide chain comprised of 585 amino acids. It contains 17 disulfide bridges. Protease enzymes break down this protein into three domains with different functions. The half-life of albumin is about 20 days, and then it is reduced by the endothelium of blood capillaries. A small amount of albumin penetrates into urine or is lost by diffusion into the alimentary canal.

Albumin is a transport protein for thyroid hormones (thyroxine, triiodothyronine), non-esterified fatty acids, unconjugated bilirubin, heme, steroid substances, plasma tryptophan and some minerals (such as calcium, magnesium and zinc). Furthermore, it binds a large number of drugs (such as penicillin, digoxin and salicylates). Another important function of albumin arises from its high plasma concentrations (35–53 g/L) and relatively low molecular weight (68 kDa). Albumin participates in the maintenance of plasma oncotic pressure (accounting for more than 75% of it).

The glycation of albumin, glycation sites and their biological impact on cell physiology has been widely studied [29–31]. In our previous work, we developed an open tubular-CEC method using a novel stationary phase to separate tryptic peptides of native and glycated BSA [32]. We also employed LC-MS/MS and CE-MS/MS techniques to study and identify glycation (carboxymethylation) sites within the BSA molecule [33]. Here we present a novel pyrraline-like structure and its location, which has been identified in BSA and HSA glycated with ribose using high-resolution nLC-MS/MS and verified by CE-MS.

2 Materials and methods

2.1 Sample preparation

2.1.1 Preparation of glycated and control BSA and HSA samples

BSA and HSA were dissolved in phosphate buffer (0.2 mol/L NaH_2PO_4; pH 7.4) to a final concentration of 1 mg/mL. These solutions were incubated (at 37°C, 7 days) with ribose (0.1 mol/L in the final solution). BSA and HSA control samples were prepared in a similar way, only without added ribose. Due to the contamination which could occur during incubation, sodium azide and a thin layer of toluene were added to each sample. Dialysis followed the incubation and lasted 24 h.

2.1.2 Reduction and alkylation (carboxymethylation) of cysteine

A total of 0.5 mL of pH 8.4 buffer (6 mol/L guanidine-HCl; 1.2 mol/L Tris-HCl; 2.5 mmol/L Na_2EDTA) was added to a 5 mg lyophilized sample of BSA. The reduction was performed by adding 25 μL DTT at a concentration of 1 mol/L. Samples were incubated for 30 min at 65°C. The carboxymethylation was then done using 60 μL iodoacetic acid at a concentration of 1 mol/L (incubation at room temperature, 40 min in the dark). The reaction was stopped by adding 15 μL of 1 mol/L DTT.

2.1.3 Enzymatic digestion of BSA and HSA by trypsin

The BSA and HSA samples were desalted on the Econo-Pac 10 DG columns (Bio-Rad Laboratories, Hercules, CA, USA) and lyophilized. Desalted samples were diluted to a concentration of 5 mg/mL with 20 mmol/L ammonium bicarbonate buffer (pH 7.8) and treated with trypsin (1:50 enzyme:substrate ratio). The incubation was done at 37°C for 24 h. Blank samples were prepared by incubating the enzyme solution alone under identical conditions.

2.2 Analysis of tryptic digests with LC-MS/MS

For analyses, the samples were diluted 100-fold and 5 μL of the sample were injected.

The nano-HPLC apparatus used for protein digest analysis was a Proxeon Easy-nLC (Proxeon, Odense, Denmark) coupled to a MaXis QTOF mass spectrometer with ultrahigh resolution (Bruker Daltonics, Bremen, Germany) by nano-electrosprayer. The nLC-MS/MS instruments were controlled with the software packages HyStar 3.2 and micrOTOF-control 3.0. The data were collected and manipulated with the software packages ProteinScape 3.0 and DataAnalysis 4.0 (Bruker Daltonics).

Five microlitres of the peptide mixture were injected into an NS-AC-11-C18 Biosphere C18 column (particle size: 5 μm, pore size: 12 nm, length: 150 mm, inner diameter: 75 μm) with a NS-MP-10 Biosphere C18 precolumn (particle size: 5 μm, pore size: 12 nm, length: 20 mm, inner diameter: 100 μm), both manufactured by NanoSeparations (Nieuwkoop, Holland).

The separation of peptides was achieved via a linear gradient between mobile phase A (water) and B (ACN), both containing 0.1% v/v formic acid. Separation was started by running the system with 5% mobile phase B, followed by a gradient elution to 30% B at 70 min. The next step was a gradient elution to 50% B in 10 min., and then a gradient to 100 % B in 8 min. Finally, the column was eluted with 100% B for 2 min. Equilibration before the next run was achieved by washing the column with 5% mobile phase B for 10 min. The flow rate was 0.25 μL min and the column was held at ambient temperature (25°C).

On-line nano-ESI (easy nano-ESI) in positive mode was used. The ESI voltage was set at +4.5 kV, scan time: 1.3 Hz. Operating conditions: drying gas (N_2): 1 L/min; drying gas temperature: 160°C; nebulizer pressure: 40 kPa. Experiments were performed by scanning from 100 to 2200 m/z. The reference ion used (internal mass lock) was a monocharged ion of $C_{24}H_{19}F_{36}N_3O_6P_3$ (m/z 1221.9906). Mass spectra corresponding to each signal from the total ion current chromatogram were averaged, enabling an accurate molecular mass determination. All LC-MS and LC-MS/MS analyses were done in duplicate.

2.3 Statistical evaluation of MS data for scheduled precursor list (SPL) generation

The profile MS chromatograms were measured for five non-glycated (control) and five glycated samples of BSA and HSA. The data sets were then statistically evaluated (*T*-test) for changes in ion intensities using the MS-*T*-test option in ProfileAnalysis software (Bruker Daltonics). The parameters for the statistical evaluation of MS data were: advanced bucketing: yes, get parameters from time alignment: yes, normalization: quantile, value count of bucket: ≥5, value count of group attribute within bucket: ≥5, allow empty group attributes: yes.

Finally, the SPLs for ions that were only found in the glycated data sets were generated and used to measure the MS/MS spectra.

2.4 Identification of unknown mass increases

The MS/MS data were searched for possible known modifications previously described by others. Several good quality spectra that had not been assigned to any particular peptide during the first database search for known modifications were then evaluated manually for the mass increase. The software ChemCalc (http://www.chemcalc.org/mf_finder/mfFinder_em_new) was used to assign molecular formulae to the exact mass increases. The new molecular formulae were then added to a MASCOT server and a new search on the same data was performed to find other possible modification sites.

2.5 Database searching

Data were processed using the software ProteinScape. Proteins were identified by correlating the tandem mass spectra of BSA and HSA samples to the IPI bovine and IPI human databases, respectively, using the MASCOT search engine (http://www.matrixscience.com).

2.6 Conditions for CE-MS analyses

Separations were performed at a voltage of 15 kV, samples of BSA were injected hydrodynamically (5 kPa; 60 s). Formic acid (0.25 mol/L) was used as the BGE. An Agilent Technologies 7100 instrument was coupled with a grounded needle and a sheath liquid (5 mmol/L ammonium acetate/isopropanol (1:1, v/v), flow rate 4 μL/min) to the MaXis mass spectrometer (Bruker Daltonics). The following conditions were applied: drying gas N_2: 8 L/min; drying gas temperature: 150°C; nebulizer pressure: 50 kPa. Before the first analysis, the capillary was washed with: 1 mol/L NaOH; water; 1 mol/L HCl; water; buffer. Each washing step lasted 20 min. Between runs, the capillary was only rinsed with the running buffer (5 min).

3 Results and discussion

High-resolution MS/MS enables the peptide/protein modifications caused by glycation to be studied, in our case monotopic modifications of lysine.

3.1 Evaluation of LC-MS data and SPL creation

The statistical evaluation of LC-MS chromatograms of five glycated and five non-glycated samples revealed a great

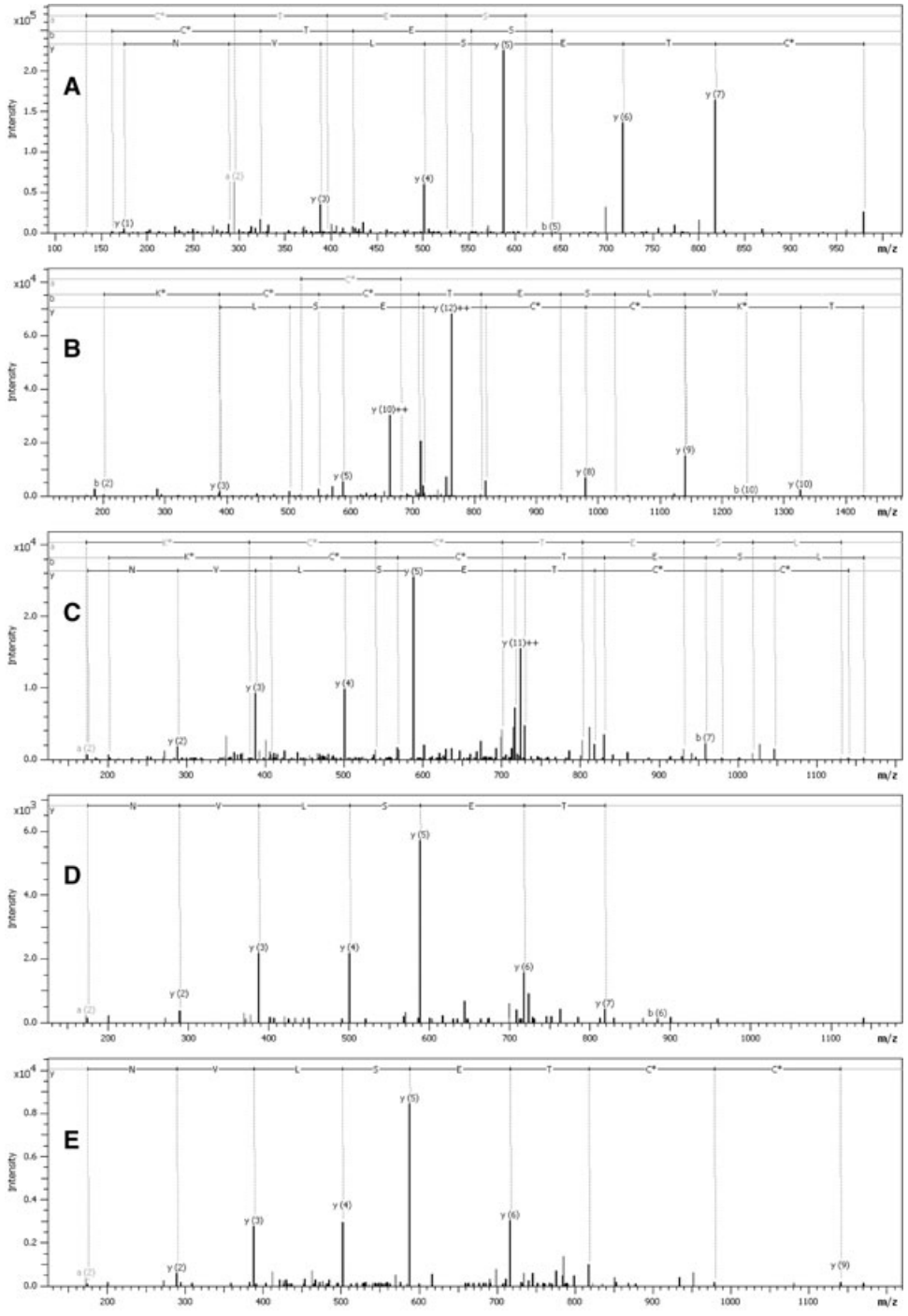

Figure 1. MS/MS spectra of (A) non-glycated peptide and peptides modified with (B) CML, $\Delta M = 58.001$; (C) C_5H_2O, $\Delta M = 78.010$; (D) Schiff base ($C_5H_8O_4$), $\Delta M = 132.038$; and (E) unknown modification, $\Delta M = 218.074$.

number of ions (precursors) that originated from glycation. These ions were only found in the glycated data set. The precise masses of the precursor ions along with their retention time served as SPLs for subsequent MS/MS experiments.

As for glycated BSA, 305 precursor ions (196 for HSA) that were not found in the control sample were observed and selected for MS/MS. An MS/MS search was performed using the SPL. Some of the most abundant ions were assigned to peptides containing CML.

3.2 Manual interpretation of unassigned spectra – identification of unknown modifications

After the exclusion of peptides modified by known AGEs found in the previous step, three good-quality unassigned MS/MS spectra that exhibited similarities to the spectra of the VTKC*C*TESLVNR peptide were evaluated manually for mass shift. The spectra of C*C*TESLVNR and VT**K**C*C*TESLVNR peptides, where **K** represents CML (ΔM:

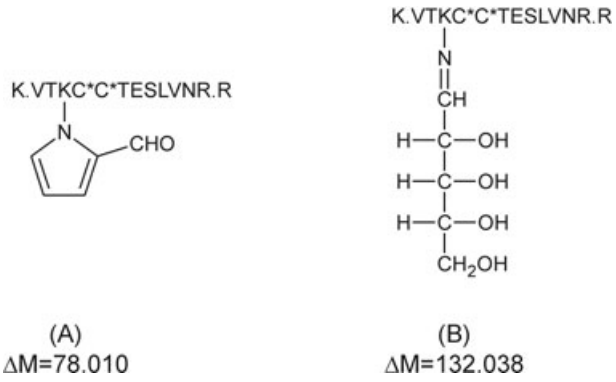

Figure 2. Schematic drawings of proposed structures of VTKC*C*TESLVNR peptide modified with (A) pyrraline-derived advanced glycation product (C_5H_2O), $\Delta M = 78.010$ and (B) Schiff base ($C_5H_8O_4$), $\Delta M = 132.038$. C* indicates carboxymethyl cysteine. Please note that the *N* in the cyclic structure originates from lysine (K) in the peptide structure.

58.001) as well as with three other unknown modifications with ΔM: 78.010, 132.038 and 218.074, are presented in Fig. 1.

High-resolution MS and MS/MS enabled us to determine the molecular formulae of these unknown modifications and propose the structure of two of them.

The first one with ΔM: 78.010 can be unambiguously assigned to the formula C_5H_2O (a modification most probably derived from pyrraline) based solely on its exact mass. It has been known for a long time that pyrraline is found in proteins glycated with glucose. It is therefore very likely that a glycation with ribose (pentose) will result in a similar structure with only five carbon atoms (Fig. 2A) missing one CH_2OH group.

The second modification with ΔM: 132.04 can be assigned to a Schiff base (Fig. 2B).

As for the last ΔM: 218.074, the possible molecular formulas include, among others, $C_{16}H_{10}O$ and $C_9H_{14}O_6$ with very similar theoretical ΔM, making it impossible to propose a structure.

3.3 Localization of other sites modified by newly proposed modifications

The new molecular formulae were added as new variable modifications into the MASCOT server and a new search was performed to see whether there are other modified sites within the BSA molecule. We found that the peptide KVPQVSTPTLVEVSR had also been modified with the same types of modifications.

3.4 Confirmation of the presence of new AGEs in glycated HSA by LC-MS/MS

In order to confirm the above-mentioned results, we also glycated HSA in addition to BSA. A comparison of the results for the BSA and HSA samples is presented in Tables 1 and 2.

Finally, raw profile MS spectra were manually searched for these modifications. We found that in some cases the signal was too low for the precursor ions to be fragmented, yet we found the exact masses for all newly discovered modifications (see the results in Table 2 with MASCOT score "N/A").

3.5 CE

The presence of novel compounds was further confirmed using CE coupled with high resolution MS spectrometer. Six of the most abundant precursor ions, that is, *m*/*z*: 516.235, 763.845, 620.009, 573.322, 591.332 and 566.653 were found in CE-MS electrophoreograms of a glycated BSA sample, confirming the presence of all newly found modification types. Unfortunately, but not surprisingly, the intensity was not sufficient to obtain quality MS/MS spectra, so the identification was based on accurate mass.

4 Concluding remarks

The glycation of proteins leads to a series of AGEs. Some of these AGEs serve as biomarkers for various diseases. Typical examples are CML and pentosidine, whose plasma levels were found to be significantly increased in diabetic patients and were proved to be potential biomarkers for type 2 diabetic retinopathy [34]. Another example is argpyrimidine, which is a useful biomarker of methylglyoxal-mediated arginine modifications in the lens and other tissues [35]. Glucosepane is one of the markers of aging and longevity in the long-lived Ansell's mole-rat [36].

To date, only a limited number of AGEs have been described, mostly originating from glucose. A search for new possible AGEs is therefore of great importance.

The identification of new AGEs is often complicated due to their very low concentration in in vivo samples. Therefore, the search for new AGEs often has to start with in vitro experiments using relatively high concentration of saccharide(s). Even then, the concentration of the resulting compounds is very low. Luckily, the high-resolution MS/MS allows us to unambiguously assign low mass increases to molecular formulae without having to isolate them and study by other techniques.

For our experiments, we have used ribose which was previously proved to react with albumins easily and rapidly and its glycation products were not described yet.

We have found that the glycation of albumins with ribose leads to a series of AGEs. In this study we used high resolution MS and MS/MS to identify two new mass increases belonging to new AGEs and we also proposed molecular formula for one of these new modifications. The Schiff base was also identified. The presence of these newly discovered modifications was confirmed in both BSA and HSA samples glycated with ribose.

The first modification with ΔM: +78.010 can be attributed to the molecular formula C_5H_2O and has a structure similar to a pyrraline, a glucose-derived AGE. The second

Table 1. Glycation sites and related peptides and modifications found in glycated BSA

Location	Peptide	*m/z*	*z*	Modification of lysine			MASCOT score
				$\Delta M_{observed}$ (Da)	$\Delta m/z$ (ppm)	Mol. formula	
K: 498	K.VT**K**C*C*TESLVNR.R	516.2345	3+	78.01026	−0,20	C_5H_2O	59.8
		763.8432	2+	58.00099	−2.94	CML	73.4
		509.5646	3+	58.00056	−3.22	CML	47.5
		534.2436	3+	132.0376	−2.93	Schiff base	59.4
		562.9224	3+	218.0740	N/A	Unknown	59.6
K: 437	R.**K**VPQVSTPTLVEVSR.S	620.0094	3+	218.0743	N/A	Unknown	68.4
		573.3203	3+	78.00659	−2.10	C_5H_2O	58.4
		591.3311	3+	132.0394	−1.64	Schiff base	64.5
		566.6517	3+	58.00115	−2.54	CML	50.3
K: 463	R.C*C*T**K**PESER.M	416.1621	3+	78.00920	−1.09	C_5H_2O	44.4

K indicates modified lysine, C* indicates carboxymethyl cysteine, CML indicates carboxymethyl lysine ($C_2H_2O_2$).

Table 2. Glycation sites and related peptides and modifications found in glycated HSA

Location	Peptide	*m/z*	*z*	Modification of lysine			MASCOT score
				$\Delta M_{observed}$ (Da)	$\Delta m/z$ (ppm)	Mol. formula	
K: 499	R.VT**K**C*C*TESLVNR.R	509.5649	3+	58.00146	−2.63	CML	64.7
		516.2334	3+	78.00696	−2.33	C_5H_2O	48.9
		562.9226	3+	218.0746	N/A	Unknown	44.8
		763.8434	2+	58.00139	−2.68	CML	N/A
		534.2433	3+	132.0367	−3.49	Schiff base	N/A
K: 438	K.**K**VPQVSTPTLVEVSR.N	573.3210	3+	78.00905	−0.88	C_5H_2O	51.9
		620.0093	3+	218.0740	N/A	Unknown	N/A
		566.6518	3+	58.00145	−2.37	CML	N/A
		591.3309	3+	132.0388	−1.98	Schiff base	N/A

K indicates modified lysine, C* indicates carboxymethyl cysteine, CML indicates carboxymethyl lysine ($C_2H_2O_2$).

modification with ΔM: +132.038 has been identified as a Schiff base.

There was also one additional mass increase (ΔM: +218.074) of an unknown structure.

We have demonstrated that there are at least two lysine locations within the albumin sequence that are prone to glycation. Our previous study showed that these positions were prone to carboxymethylation [33]. Here we have found that in addition to CML, these sites were modified by a series of other AGEs, which indicates that they are highly reactive. For BSA, these lysines are at positions 437 and 498 (for HSA, the positions are: 438 and 499).

This work was supported by the Czech Science Foundation (Grants Nos. P206/12/0453 and 203/08/1428), the Academy of Sciences of the Czech Republic support for long-term conceptual development of research organization RVO: 67985823, which is gratefully acknowledged.

The authors have declared no conflict of interest.

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