Research Article

Off-line liquid chromatography-MALDI by with various matrices and tandem mass spectrometry for analysis of glycated human serum albumin tryptic peptides

Annunziata Lapolla[1], Francesco L. Brancia[2], Jessica Bereszczak[2], Domenico Fedele[1], Lorenzo Baccarin[1], Roberta Seraglia[3] and Pietro Traldi[3]

[1] Dipartimento di Scienze Mediche e Chirurgiche, Università di Padova, Padova, Italy
[2] Shimadzu Research Laboratory, Manchester, UK
[3] CNR-ISTM, Padova, Italy

Advanced glycation end-product (AGE)/peptides, arising from *in vivo* digestion of glycated proteins, are biologically important compounds, due to their reactivity against circulating and tissue proteins. For information on their possible structure, *in vitro* glycation of HSA and its further enzymatic digestion were performed. The resulting digestion product mixture was analysed directly by MALDI MS with various matrices [2,5-dihydroxy benzoic acid (DHB) and α-cyano-4-hydroxy cinnamic acid (CHCA)]. Alternatively, offline microbore LC prior to MALDI analysis was used, and showed that 63% of the free amino groups prone to glycation are modified, indicating the contemporary presence of unglycated peptides. This result proves that, regardless of the high glucose concentration employed for HSA incubation, glycation does not go to completion. Further studies showed that the collisionally activated decomposition of singly charged glycated peptides leads to specific fragmentation pathways, all related to the condensed glucose molecule. These unique product ions can be used as effective markers to establish the presence of a glucose molecule within a peptide ion.

Keywords: AGE/peptides / Diabetes / Glycation / Human serum albumin / Off-line LC-MALDI

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1 Introduction

The nonenzymatic protein glycation observed in diabetes has been related to the high glucose concentration, typical of the disease, in biological fluids [1–3]. This reaction takes place between protein free amino groups and sugars, accordingly to the Maillard reaction pattern [4, 5]. Glycated proteins stimulate a macrophage response, with subsequent internalization and digestion: the resulting glycated peptides are usually called advanced glycation end-product/peptides (AGE/peptides) and their important pathological role has been the object of many researches.

AGE/peptides can interact with circulating and tissue proteins. In the former case, leading to protein modifications, this process influences their functionality; in the latter case, this reaction is considered an explanation for the tissue modifications typical of long-term diabetic complications [6, 7]. AGE/peptides are also excreted by kidneys and consequently, in renal failure, their concentrations increase, leading to the activation of more severe pathologies [8–12].

Due to the glycation yield, as well as to the complex mechanisms responsible for glycated protein digestion, the level of AGE/peptides in plasma is, in principle, very low. Thus, in view of the intrinsic complexity of plasma, direct identification of AGE/peptides is certainly a difficult task.

In previous researches, some preliminary *in vitro* experiments were carried out in order to find the best analytical procedure for AGE/peptide identification. For this aim glycated peptides originating from proteolytic digestion of *in vitro* glycated bovine (BSA) and HSA was studied [13–16], based on LC/MS analysis of low molecular weight peptides generated by enzymatic digestion and MALDI/MS of the whole protein digest. A series of glycated peptides was identified.

Correspondence: Professor Annunziata Lapolla, Dipartimento di Scienze Mediche e Chirurgiche, Via Giustiniani n 2, I-35100 Padova, Italy
E-mail: annunziata.lapolla@unipd.it
Fax: +39-049-8216838

When applied to *in vivo* samples (plasma samples of diabetic and nephropathic patients), the same methods were unable to identify glycated peptides originating from *in vivo* glycated HSA. However, in MALDI/MS conditions, clearcut differences were observed among the low molecular weight plasma peptide fractions from diabetic and nephropathic patients and healthy subjects [17, 18]. These results were explained by: (i) digestion mechanisms occurring in *in vivo*, which differ greatly from those occurring in *in vitro* enzymatic digestion; (ii): the complexity of plasma means that the detection of AGE/peptides is hampered by the limited dynamic range of the MS approach.

The positive results obtained by MALDI only revealed the presence of specific low molecular weight peptides in the case of plasma samples from pathological patients, although no structural information was obtained, due to the impossibility of carrying out any MS/MS experiment, with the MALDI equipment used.

In this paper, we present a MALDI-based method which links off-line microbore RP LC with tandem mass spectrometric analysis on a TOF/curved field reflectron tandem mass spectrometer. Distinctive collisionally generated fragment ions observed for glycated peptide ions unequivocally identified glycated peptides in complex mixture, and consequently represent an effective analytical tool to study the presence of AGE/peptides in plasma samples.

2 Materials and methods

2.1 Materials

All chemicals were of analytical grade. MALDI matrices α-cyano-4-hydroxy cinnamic acid (CHCA) and 2,5-dihydroxy benzoic acid (DHB) were supplied by LBL (LaserBio Labs, France). TFA and HSA were supplied by Sigma (Poole, UK). Organic solvents acetone, methanol, ACN and water were obtained from Rathburn (Walkerburn, Scotland).

2.2 Glycation of HSA

In vitro glycation of pure HSA was carried out with glucose in pseudophysiological conditions according to a published procedure [16]. Briefly, HSA (Sigma, St. Louis, MO, USA; 100 mg/mL in 0.01 M phosphate buffer, pH 7.4, containing 5 mM toluene as bacteriostatic) was incubated with 2 M D-glucose (Sigma) at 37°C for 14 days. After incubation, HSA was separated from the solution by centrifugation through a Centricon-50 membrane (Millipore Corporation, Bedford, MA, USA; MW cut-off 50 000 Da) at 5000 × *g* for 1 h, dialysed extensively against distilled water (MW cut-off 50 000, Spectrum Laboratories) and then lyophilized. Control HSA was incubated in the same conditions without the addition of glucose.

2.3 Enzymatic digestion

Glycated HSA (0.25 mg) was dissolved in 320 μL of 50 mM NH_4HCO_3 buffer solution (pH 8.3). After the addition of 3.7 μL of a 45 mM solution of DTT, the mixture was heated at 50°C for 15 min. About 3.7 μL of a 100 mM solution of iodoacetamide was added to the mixture and the solution was maintained at room temperature in the dark for 15 min. It was then incubated with trypsin (50 μL of a 100 ng/μL solution, substrate to enzyme ratio = 50 : 1 w/w) overnight at 37°C. The reaction was stopped with 20 μL of 10% TFA. The final peptide concentration was about 10 pmol/μL.

2.4 LC and MALDI sample collection

RP capillary HPLC was performed on an LC-2010 system (Shimadzu, Kyoto, Japan). Differentially labelled peptide solutions (1 μL) were prepared with 0.05% v/v aqueous TFA and loaded using a Cheminert C1-1006 six port valve (VICI, USA). Components were separated on a capillary LC Packing C_{18} Pep MAP 100 column (0.3 mm id × 10 mm). In gradient mode, the mobile phase composition was 0.05% v/v TFA for the aqueous phase and 0.05% TFA v/v and ACN (8 : 2 v/v) for the organic phase. LC-$10AD_{VP}$ HPLC pumps (Shimadzu) were used to deliver the solvent at a flow rate of 200 μL/min. The composition of %B was increased linearly for 50 min from 0 to 55%. The gradient profile was controlled by LCMS Solution 2.0 Software. The UV response of the column eluent was monitored at a wavelength of 220 nm. The system was configured to perform a precolumn split. The sample, eluted at an estimated flow rate of 5 μL/min, was delivered to an online AccuSpot LC-MALDI spotting robot system (Shimadzu). The eluent was mixed with a saturated matrix solution prepared in 50% v/v ACN acidified with 0.1% v/v TFA. Ten spots of 1 μL each were deposited onto the MALDI target every minute.

2.5 MALDI-TOF analysis

All positive MALDI-TOF mass spectra were acquired on an AXIMA-CFR plus instrument (Shimadzu Biotech, Manchester, England) equipped with a 1.2 m drift tube. MALDI of peptides was produced by pulses of UV light (λ = 337 nm, 3 ns pulse width) generated by a nitrogen laser with a maximum pulse rate of 10 Hz. Spectra were collected in reflectron mode using a delayed extraction of 150 ns. The accelerating voltage was set to +20 kV. The reflectron voltage was set to +25 kV. The spectra were the sum of at least 100 profiles on the same sample spot. Each profile was the result of two consecutive single laser pulses. Acquisition and data processing were controlled by Launchpad software version 2.7 (Kratos Analytical). The TOF was externally calibrated with an equimolar mixture of bradykinin, neurotensin and human adrenocorticotropic hormone ACTH 18–38. Data analysis was performed using raw data.

2.6 MALDI MS/MS

MS/MS spectra were acquired on a prototype MALDI mass spectrometer based on the Axima CFR instrument (Shimadzu Biotech), with the addition of a high resolution ion gate and a differentially pumped collision cell. Samples are introduced by a microtiter format metal plate. A nitrogen laser was used to ionize the MALDI samples. The pulsed extraction ion source typically accelerated the ion beam to 20 keV. A novel ion gate design was used, providing a precursor ion resolution of up to 500 (FWHM). The collision cell could be flooded with a choice of collision gases, typically helium. The pressure could be regulated by means of a needle valve. Typical collision cell pressures were in the range of 10^{-5}–10^{-4} mbar, as estimated from the differential pumping regime by a direct measurement in the differentially pumped chamber. The above collision cell pressures were optimized for an average single collision of a typical peptide molecule. The collision cell was grounded, whereas the incoming ion beam was typically accelerated to 20 keV, resulting in centre-of-mass collision energies of the order of magnitude of a few tens of electron volts, assuming helium as the collision gas. The path length through the high-pressure region of the collision cell was approximately 15 mm. The instrument had a curved-field reflectron, which allowed the efficient collection of all fragment ions, including those produced from metastable decay (PSD mode) as well as CID occurring in the collision cell (CID mode). The curved-field reflectron efficiently reflects precursor and fragment ions onto the surface of a fast microchannel plate detector. Signals were collected by a transient recorder with a sampling time of 0.5 ns and processed by a computer system.

3 Results

HSA was glycated in pseudophysiological conditions at 37°C with 2 M glucose for 14 days. Glycated HSA was digested with trypsin and the digested products were analysed by MALDI.

Two different matrices were used: DHB and CHCA, both widely employed for peptide mixture analyses. The resulting spectra of the digestion mixture from glycated HSA are reported in Figs. 1a and b. Clearly, DHB gives the best results: a higher number of digestion products is observed.

To overcome possible suppression effects, which may potentially lead to only a partial view of peptide fingerprinting, the digestion mixture containing glycated peptides was separated by RP LC prior to MALDI analysis. The resulting chromatograms are shown in Fig. 2. This approach allows the detection of unmodified tryptic digest fragments providing 82% coverage (see Table 1).

The MS/MS spectra of glycated peptides show peculiar decomposition pathways. As in the example shown in Fig. 3 (LK*CASLQK*FGER diglycated peptide), losses of neutral species of 120 and 162 Da are observed.

4 Discussion

MALDI is a highly effective technique for direct analysis of both intact proteins and tryptic digestion mixtures, allowing identification of most peptides generated by enzyme action. In particular, in the field of protein glycation, mass spectrometric monitoring of albumin in uremia was described by Thornalley *et al.* [19] comparing MALDI data from *in vitro* and *in vivo* glycation. MALDI/MS study also identified the extent of glycation of HSA and related it with the reduced intramolecular movements of this protein, impairing exposure of binding sites for many drugs and natural ligands [20].

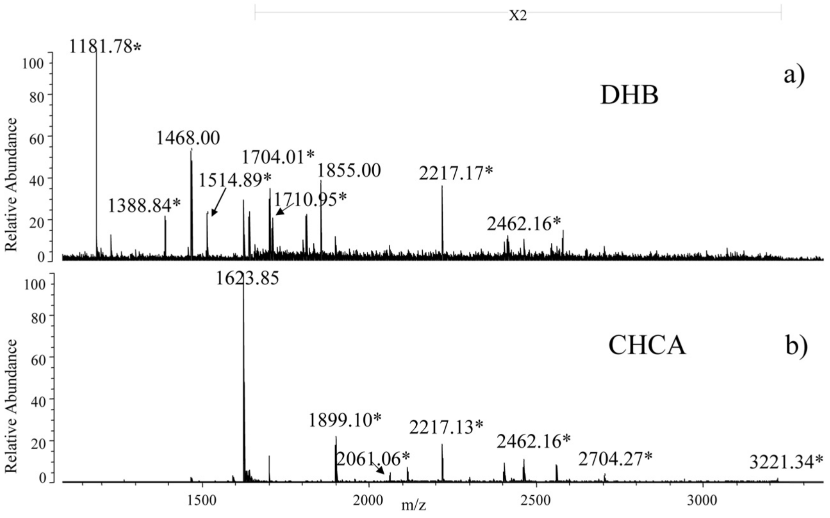

Figure 1. MALDI spectra of an unfractionated digestion mixture of glycated HSA acquired in DHB (a) and CHCA (b).

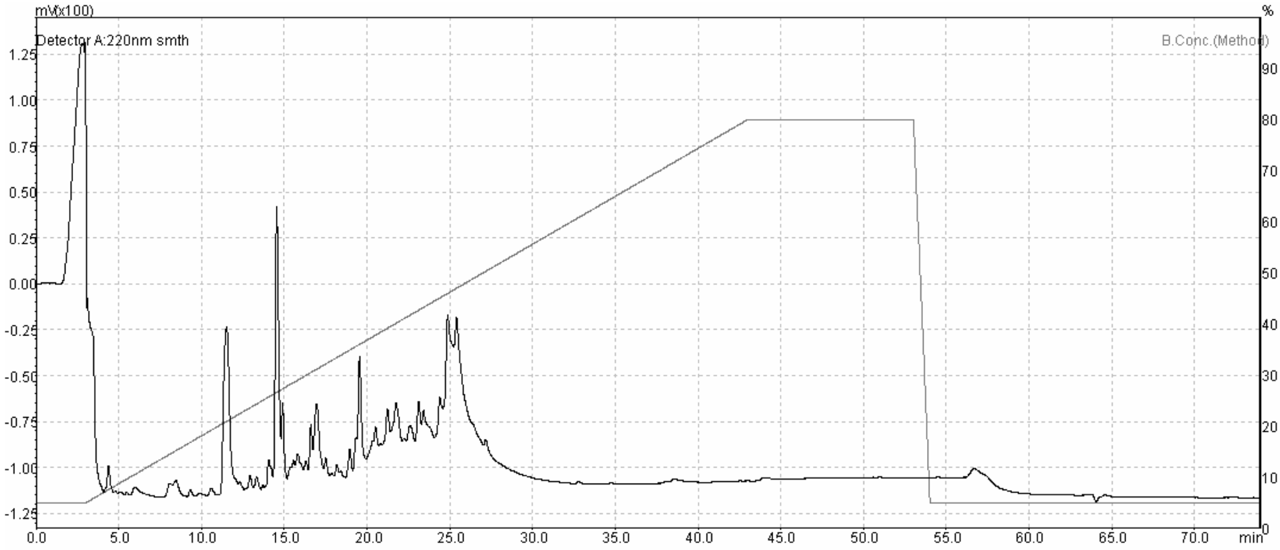

Figure 2. RP capillary LC chromatogram of the enzymatic digestion products of *in vitro* glycated HAS.

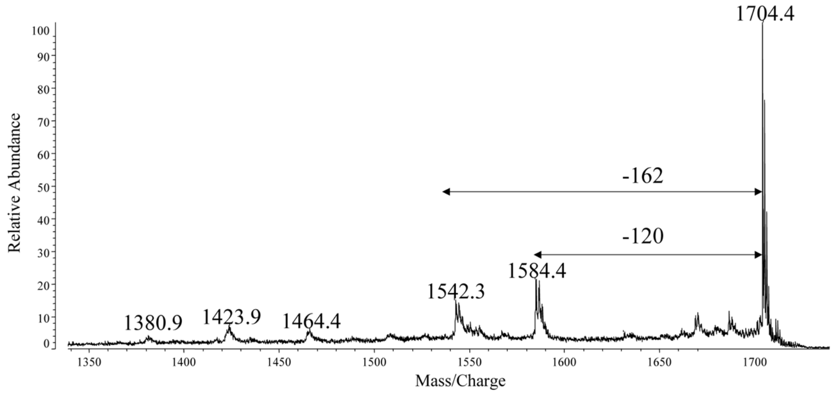

Figure 3. Product ion spectrum of glycated LK*CASLQK*FGER, analysed on a TOF/curved field reflectron tandem mass spectrometer.

More recently Ahmed and Thornalley [21] presented an interesting study on peptide mapping of HSA, modified minimally by methylglyoxal both *in vitro* and *in vivo*.

These papers are a good example of the power of MALDI/MS in investigating the products arising from protein glycation.

In previous studies [13–16] on MALDI of glycated peptides, CHCA was employed as matrix, leading to partially satisfactory results. However, looking at the nature of the species of interest, *i.e.* glycated peptides, we thought of testing the effectiveness of several different matrices.

The spectra obtained by DHB and CHCA for digestion mixtures from glycated HSA are shown in Fig. 1. Both peptide mass fingerprints are almost identical in the type of ions detected: of 45 tryptic peptide ions identified, 17 fragments contain a middle chain lysine, due to missing cleavage, providing a final 82% protein coverage. Interestingly, when the mixture obtained from proteolysis of glycated HSA was analysed by DHB, an increase in the type of ions detected was observed. As it can be seen in Fig. 1a, abundant glycated ions, which were undetected in the previous investigations [13–16], are present in the region 500–2000 Th. Conversely, using CHCA, more intense glycated peptide ions are observed in the region 2000–3300 Th. However, greater recovery of glycated tryptic peptides is obtained when DHB is employed. Identification of 15 glycation sites out of 60 amino groups available (including an additional glycated adduct at 3221.16 *m/z*, detected in CHCA) indicates that only 25% of the reaction sites undergo glycation. Fourteen unglycated peptides are still detected among the digestion products of glycated HSA. If the digestion process is extensive, one would expect a protein coverage of 75%, whereas this value was only found to be 31%, demonstrating that many unglycated peptides are missing among the digestion products of glycated HSA.

An interesting approach is now proposed for the analysis of digestion mixtures based on RP LC prior to MALDI analysis. Isolation of selected components in consecutive spots generates simpler mixtures, thus overcoming or reducing any suppression effects. A 40 min gradient yielded the chromatogram shown in Fig. 2. The sample, eluted with an estimated flow rate of 5 μL/min was delivered to an online spotting robot system. Ten spots of 1 μL each were deposited onto the MALDI target every minute. This approach meant that 49 modified peptides could be observed, as shown in Table 1. Comparison of LC-MALDI and MALDI data clearly emphasize the power of the former approach. The resulting data show that glycation took place on 39 lysine residues, and that several unglycated tryptic digest peptides are present in the spots isolated by LC. This indicates that glycation does not go to completion.

For valid identification of AGE/peptides produced in *in vivo* conditions, *m/z* determination is certainly a limited approach. A complementary, more specific, analytical tool is needed and, in this frame, the MS/MS approach, by a curved field reflectron tandem mass spectrometer, is definitely of interest.

As an example the product ion spectrum of diglycated LK*CASLQK*FGER is shown in Fig. 3, highlighting the presence of two fragments usually absent in unglycated peptides. The ions at *m/z* 1584 and 1542 originate by loss of neutral species of 120 and 162 Da, respectively. The latter corresponds to the loss of dehydrated glucose, while the former originates by cleavage of the glucose residue with loss of a $C_4H_8O_4$ neutral species.

This behaviour was observed for all glycated peptides, independently of lysine position and/or peptide dimension. In all cases, abundant ions corresponding to losses of 162 and 120 Da were observed. Consequently, these fragments may be considered a distinctive signature of the occurrence of glycation processes. In other words, without previous knowledge of any modifications, the presence of these peptides allows us to determine whether the fragment is glycated or not.

In conclusion, this study identified a series of glycated peptides originating by enzymatic digestion of *in vitro* glycated HSA (see Table 1). Their presence still needs to be tested in plasma samples from diabetic and nephropathic patients, in whom, however, glycation processes and enzymatic digestion may take place in different conditions.

Apart from AGE peptide mapping, the present work mentions some important points which must be taken into account in further studies: (i) the chemical nature of the matrix plays a significant role in affecting the quality of the resulting MALDI spectra. DHB and CHCA, both widely employed in protein/peptide analysis, give the best results with the former; (ii) the off line LC-MALDI approach led to a considerable increase in protein coverage for glycated HSA; (iii) data for glycated protein showed that, although more than 80% of the reaction sites are glycated, many unglycated peptides are still detected, indicating that glycation does not go to completion; (iv) MS/MS MALDI analysis provides significant results: in the case of glycated peptides, losses of the glucose moiety are observed proving the value of this approach to identify glycated species unambiguously.

Table 1. Summary of glycated peptide ions observed in unfractionated mixtures and after off-line LC/MALDI

Position	Glycated [M + H]$^+$ LC MALDI	Glycated [M + H]$^+$ MALDI
435–438	701.08	
258–264	951.44	
559–565	971.23	
230–236	1016.29	
461–468	1077.57	
37–44	1113.38	
559–565	1133.41 DD	
563–569	1141.39 DD	
234–242	1181.40	1181.78
161–168	1217.44	
461–469	1233.42	
461–468	1239.50 DD	
549–558	1290.41	
224–233	1300.44	
338–347	1302.40	
66–75	1311.64	
35–44	1388.58	1388.84
461–469	1395.37 DD	
373–383	1458.60	1458.88
211–221	1500.73 DD	
427–437 or 497–508	1514.54	1514.89
301–310	1515.61 DD	
222–233	1541.60	
384–396	1543.46	
247–257		1576.95 DD
427–438	1642.71	
230–242	1670.82	
206–219	1680.72	
222–233	1703.68 DD	1704.01 DD
185–198	1710.82	1710.95
438–452		1802.15
250–264	1812.81	1813.10
550–562	1814.93 DD	
184–198	1866.78	
243–257	1898.90	1899.13
89–105	2037.15	
169–183 or 170–184	2061.12	2061.06
243–257		2061.06 DD
439–456	2085.96	
106–122	2121.79	
247–264	2185.16	
397–413	2207.15	
169–184	2217.18	2217.17
342–360	2462.44	2462.13
376–396	2509.36	
470–490	2566.54	
45–65	2595.46	
525–545	2650.53	
414–434	2704.60	2704.30
76–97	2707.56 DD	
139–161	2883.71	
106–130	3121.72	
470–496		3221.16

DD: presence of two modified sites.

In the near future, the same methods will be applied to study the presence and structure of AGE/peptides in plasma samples from healthy, diabetic and nephropathic subjects.

5 References

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