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A study on human serum albumin influence on glycation of fibrinogen

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ABSTRACT

Although *in vivo* glycation proceeds in complex mixture of proteins, previous studies did not take in consideration the influence of protein–protein interaction on Maillard reaction. The aim of our study was to test the influence of human serum albumin (HSA) on glycation of fibrinogen. The isotopic labeling using [$^{13}C_6$] glucose combined with LC-MS were applied as tool for identification possible glycation sites in fibrinogen and for evaluation the effect of HSA on the glycation level of selected amino acids in fibrinogen.

The obtained data indicate that the addition of HSA protects the fibrinogen from glycation. The level of glycation in presence of HSA is reduced by 30–60% and depends on the location of glycated residue in sequence of protein.

1. Introduction

Diabetes is a widespread disease, involving about 8.3% of the world adult population. The number of deaths attributable to this disease in 2011 was approximately 4.6 million, which is a serious and growing social problem.

High glucose concentration in biological fluids has been shown to be the main cause of accelerated non-enzymatic protein glycation in diabetes [1]. This reaction consists in condensation of reducing sugars and free amino groups at the N-terminus or on lysine/arginine side chains. The glycation is initiated with the formation of an Amadori product [2], which undergoes further modification to a heterogeneous group of substances, called advanced glycation end products (AGEs) [3]. Those processes can involve circulating proteins as well as tissue proteins influencing their functions and structures. As a result, AGEs's formation is responsible for tissue modifications [4,5].

Fibrinogen is a high molecular weight (341,000 Da), dimeric glycoprotein found in blood of vertebrates, where it plays a critical role in coagulation system [6]. A monomer of this protein contains two sets of three different chains (Aα, Bβ, and γ), which six N-termini create a central, symmetric globular region ("E-region") [7,8]. This region is joined to two outer globular regions ("D regions") formed by the C-terminal parts of the Bβ and γ chains (each folding into independent domains) by two triple coiled-coil connectors. These coiled-coil connectors are stabilized at their beginning and end by two disulfide bridge rings [9,10]. At the end of each coiled-coil, the A alpha chains' reverse direction forms a fourth coiled helix. The carboxyl-terminal regions of these chains (alpha C regions) form compact alpha C-domains. According to literature data [11], two alpha C-domains interact intramolecularly with each other and with the central E region preferentially through its N-termini of B beta chains.

In the last years, there is a great interest in fibrinogen as a target for Maillard reaction. A study was performed by Svensson et al. on using isotope labeling of this protein by [^{14}C-acetyl] salicylic acid and [^{14}C] glucose *in vitro* [12]. They found two glycated lysines (K163 in the beta chain and K101/K111 in the gamma chain), and these are within the "plasmin-sensitive" coiled–coil regions. They could not find any interaction between aspirin and glucose in binding to fibrinogen, on the grounds that aspirin and glucose bind to different lysines. Furthermore, several of these sites have a potential importance for cross-linking by FXIIIa [13,14], and may thus also impact on the fibrin network.

The literature data discuss the problem of glycation of many proteins under *in vitro* conditions. In those studies proteins are treated as independent, non-interacting systems. In real situation Maillard reaction affects extremely complex mixtures of interacting proteins. At the moment there are only limited data concerning the influence of possible interactions between proteins during this process. Those data suggest that certain proteins may have an impact on glycation and possibly other non-enzymatic modifications of e.g. fibrinogen [15] or low-density lipoprotein [16].

Herein we studied the effect of human serum albumin (HSA) on glycation of fibrinogen in a model system. Albumin is one of the proteins which are heavily glycated in diabetes (high abundance, relatively longer half-life time, larger number of K and R residues). Bhonsle et al. [15] proved that low levels of albumin are associated with increased glycation of other proteins. The results of this study should provide a better understanding of the influence of HSA on

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the fibrinogen glycation at the level of particular amino acid residues.

2. Materials and methods

2.1. Reagents

Reagents (including isotopicaly labeled $^{13}C_6$ glucose; 99% ^{13}C) and solvents purchased from Sigma–Aldrich were used without further purification. The fibrinogen from human plasma was obtained from Calbiochem.

2.2. In vitro glycation of fibrinogen

A. Sample of fibrinogen (Calbiochem) was glycated according to the Boratynski method [17,18] using an equimolar mixture of [$^{12}C_6$] glucose and [$^{13}C_6$] glucose. Samples were mixed with these sugars and dissolved in water to give a protein to sugar molar ratio of 1:100; 1:370; 1:500; 1:1000. The samples were lyophilized. The dry lyophilizate was heated at 80 °C for 25 min.
B. Two samples of fibrinogen 5 mg each were incubated: one in 1 ml 0.5 M $^{12}C_6$-glucose and containing 1 mg of HSA and the other in 1 ml 0.5 M $^{13}C_6$-glucose at 37 °C for 72 h. This method was based on Glycation Isotope Labeling (GIL) [19,20]. Equal volumes of these samples were mixed before further processing.

2.3. Ultrafiltration

The obtained material was centrifuged through a Centricon-10 membrane for 20 min in order to separate the low molecular weight fraction from the high molecular weight one.

2.4. Reduction

A. The glycated protein (1 mg) was dissolved in 50 mM NH_4HCO_3 buffer solution. DTT (10% in water) was added, and then the mixture was incubated at 50 °C for 30 min.
B. Another procedure was also applied to samples of fibrinogen glycated *in vitro*. The glycated protein (1 mg) was dissolved in 50 mM NH_4HCO_3 buffer solution. DTT 50 mM (b1) or 100 mM (b2) was added, and then the mixture was incubated at 60 °C for 30 min .

2.5. Hydrolysis

The trypsin solution (from bovine pancreas) was added to the mixture of reduced protein to obtain the 1:10 (a) or alternatively 1:100 (b) enzyme: substrate mass ratio. The mixture was incubated at 37 °C for 24 h. Digestion was terminated by the addition of 10 μl of trifluoroacetic acid. Then samples were subjected to LC–MS analysis.

After hydrolysis (b) the sample with [$^{12}C_6$] D-glucose from GIL method was added to sample with [$^{13}C_6$] D-glucose in 1:1 M ratio. Then combined sample was subjected to LC–MS analysis.

2.6. LC-MS

The LC-MS analysis was performed in the Laboratory of Mass Spectrometry at the Faculty of Chemistry, University of Wroclaw using Agilent 1200 HPLC system coupled to micrOTOF-Q mass spectrometer (Bruker Daltonics, Germany). For separation an Aeris PEPTIDE, Phenomenex (50 × 2.1 mm, 3.6 μm) column was used, with two elution gradients of 0–100% B in A (*A* = 0.1% HCOOH in water; B = 0.1% HCOOH in acetonitrile) over 32 min and over 62 min (flow rate 0.1 ml/min or flow rate 0.05 ml/min, room temperature).

2.7. LC-MS/MS

The LC-MS/MS analysis was performed using the same equipment in the Laboratory of Mass Spectrometry at the Faculty of Chemistry with elution gradient of 0–100% B in A (*A* = 0.1% HCOOH in water; *B* = 0.1% HCOOH in acetonitrile) over 62 min (flow rate 0.1 ml/min, room temperature). We used an energy of 22 eV for fragmentation.

2.8. Data analysis

The mass list generated by data analysis program (Bruker, Germany) was analyzed using our home-developed software written in JAVA. The procedure is based on searching for pairs of ions of equal abundances in which the difference of monoisotopic mass was equal to $1.003*n$, where n = the number of carbon atoms from glucose. The accepted error of mass difference was below 0.02 Da while the accepted difference of abundances below 10%. The program calculates theoretical masses of peptides obtained from in silico digestion taking in consideration the defined specificity of protease. Then it assigns the peptide sequences to peaks from the scans of the LC–MS data set and generates lists of potential glycated peptides using mass shifts characteristic for early glycation products.

An amino acids sequence of chains of human fibrinogen was in accordance with the UniProt Knowledgebase (UniProtKB). The analysis was based on isoforms alpha-E and gamma-B. The numbering of amino acids residues in sequence corresponds to the proteins with signal peptides.

3. Results and discussion

Recently we have proposed a method for detection of the glycation sites in proteins based on combination of glycation with the mixture of $^{13}C_6$ and $^{12}C_6$ glucose and LC–MS or HRMS [21,22]. This approach was further developed and applied for fast and convenient identification of low level glycation sited in recombinant antibodies [20].

The objective of the current study was an evaluation of influence of HSA on the glycation of selected moieties in fibrinogen. For this purpose, we have studied this reaction of mentioned proteins, under model *in vitro* conditions.

Our approach consisted of two related experiments. Firstly, the ^{13}C isotopic labeling combined with LC–MS was used as a tool for mapping susceptible to glycation lysine residues in fibrinogen. The first part of this study revealed the potential glycation sites in fibrinogen molecule. Basing on this information we found the amino acid residues glycated in solution with and without HSA and compared glycation efficiency in these two cases.

3.1. Identification of glycation sites

In the first experiment fibrinogen was modified by an equimolar mixture of glucose with natural isotopic composition and [$^{13}C_6$] glucose. Then the glycated samples of protein were subjected to reduction of the disulfide bridges followed by enzymatic hydrolysis using trypsin. Selecting trypsin also enabled a comparison of our results with literature data [23]. The tryptic digests were analyzed by LC–MS. Amadori products were identified on the basis of characteristic isotopic patterns [21,22] which is presented in Fig 1. The analysis of data was performed automatically using home-

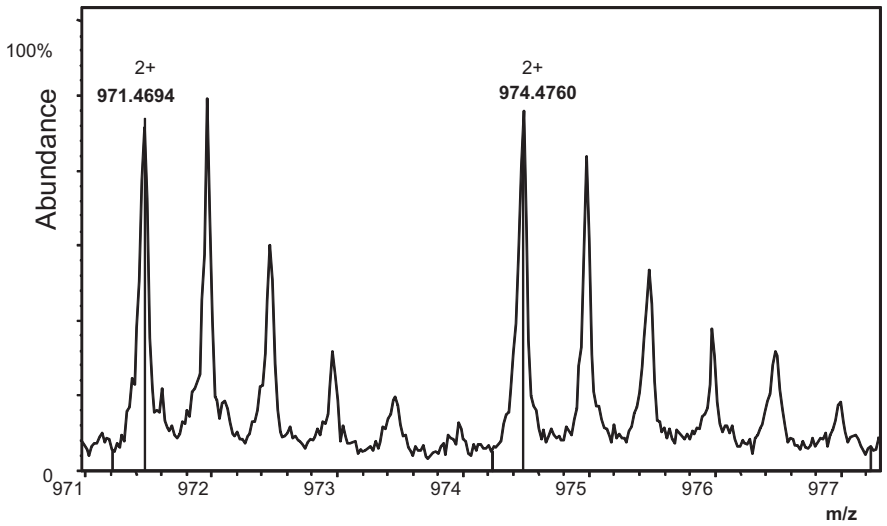

Fig. 1. Averaged mass scans for 14–15 min of LC-MS chromatogram of products obtained by enzymatic hydrolysis of fibrinogen glycated with an equimolar mixture of glucose and [$^{13}C_6$] glucose according to experimental part (procedure A). The pairs of peaks with equal abundance and distance equal to 3.009 Th correspond to the $[M+2H]^{2+}$ ion of the Amadori product [70–84] alpha 1 chain.

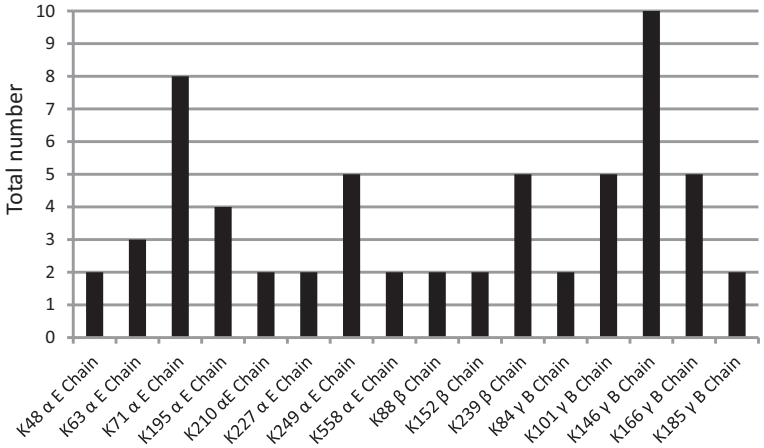

Fig. 2. The total number of identified peptide-derived Amadori products containing glycated K residues in products of tryptic hydrolysis of glycated fibrinogen. The data were shown separately for alpha E, beta and gamma B chains of fibrinogen.

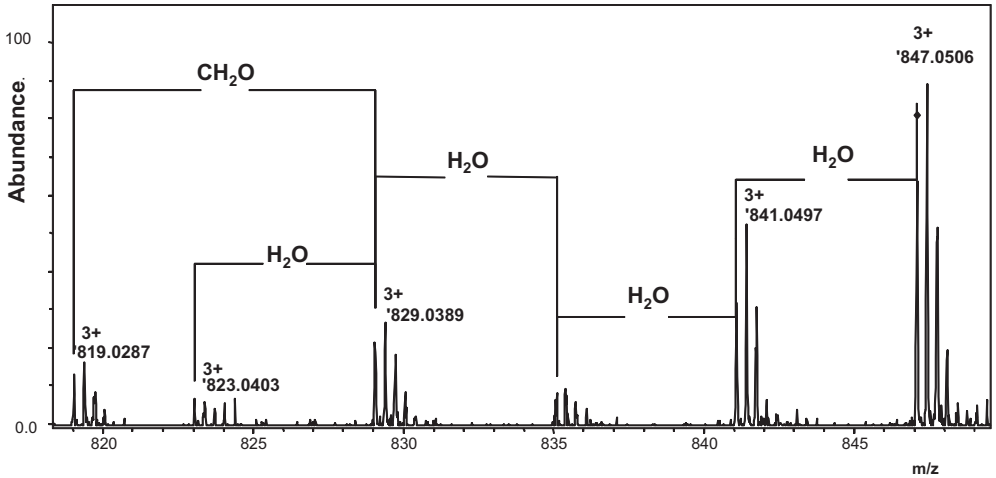

Fig. 3. Fragmentation pattern of Amadori product obtained by tryptic hydrolysis of glycated fibrinogen [225–246] beta chain. (LC-MS/MS experiment).

Table 1
Amadori products identified in differential glycation of fibrinogen: elution gradient of 0–100% B in A (*A* = 0.1% HCOOH in water; *B* = 0.1% HCOOH in acetonitrile) over 62 min (flow rate 0.05 ml/min); reduction (B), hydrolysis (B2).

m/*z*	*z*	Exp. mass of glc. peptide	Calc. mass of glc. peptide	Sequence
600,0695	4	2396,2467	2396,2448	[52–72] beta
580,0096	3	1737,0053	1737,0036	[145–157] alpha 1
731,0948	4	2920,3479	2920,3475	[548–573] alpha 1
763,3670	3	2287,0775	2287,0757	[295–313] beta
1144,5365	2	2287,0573	2287,0757	[295–313] beta
637,5639	4	2546,2243	2546,2210	[247–267] beta
660,8205	6	3958,8760	3958,8711	[154–188] gamma B
792,7832	5	3958,8769	3958,8711	[154–188] gamma B
849,7470	3	2546,2175	2546,2210	[247–267] beta
990,7237	4	3958,8635	3958,8711	[154–188] gamma B
1274,1174	2	2546,2191	2546,2210	[247–267] beta
703,6729	6	4215,9904	4216,0086	[152–188] gamma B
741,5504	5	3702,7129	3702,7175	[154–185] gamma B
1122,0751	2	2242,1345	2242,1481	[135–151] gamma B
1235,2425	3	3702,7040	3702,7175	[154–185] gamma B
732,1192	4	2924,4455	2924,4437	[89–113] gamma B
792,9773	5	3959,8474	3959,8551	[152–185] gamma B
975,8238	3	2924,4479	2924,4437	[89–113] gamma B
802,4203	2	1602,8249	1602,8327	[226–238] alpha 1
647,9809	3	1940,9192	1940,9150	[70–84] alpha 1
894,7768	3	2681,3069	2681,3106	[89–111] gamma B
971,4623	2	1940,9089	1940,9150	[70–84] alpha 1
609,0970	4	2432,3567	2432,3501	[219–238] alpha 1
646,0782	4	2580,2815	2580,2807	[191–210] alpha 1
832,3317	4	3325,2955	3325,2845	[43–69] alpha 1
654,3214	3	1959,9407	1959,9434	[244–258] alpha 1
847,0755	3	2538,2030	2538,2016	[225–246] beta
980,9706	2	1959,9255	1959,9434	[244–258] alpha 1
879,6308	5	4393,1149	4393,1197	[161–196] beta
707,5666	5	3532,7939	3532,7897	[191–218] alpha 1
664,8371	4	2655,3171	2655,3136	[114–134] gamma B
839,4016	5	4191,9689	4191,9580	[459–491] beta
886,1125	3	2655,3140	2655,3136	[114–134] gamma B
1333,9707	3	3998,8886	3998,8732	[125–157] beta

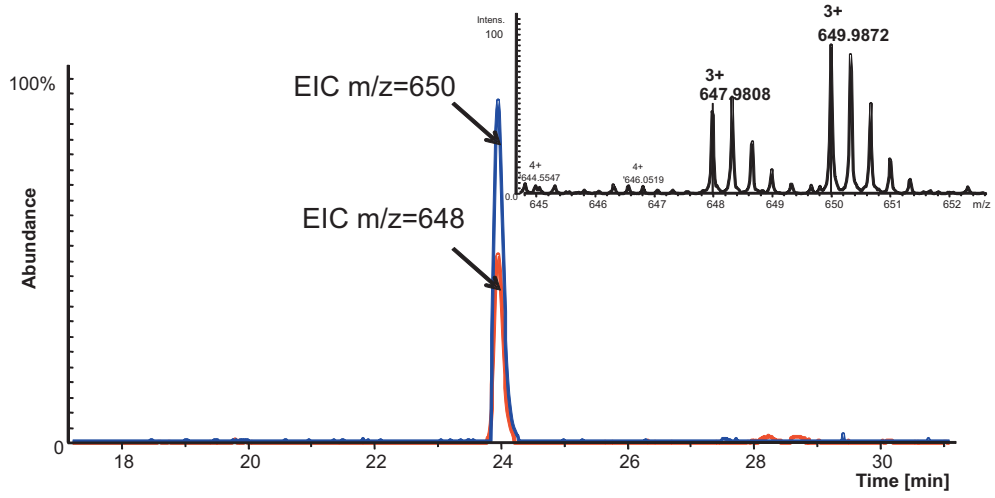

Fig. 4. Differential glycation experiment – averaged mass scans of LC-MS chromatogram of sample prepared according experimental part and extracted ion chromatograms (EIC) for *m*/*z* 650 and 648 corresponding to [70–84] alpha 1 chain of fibrinogen labeled with $^{13}C_6$ glucose and unlabeled, respectively.

developed JAVA program. The error accepted in the search was always under 10 ppm. To confirm the obtained results we used an additional criterion – the retention times of labeled and unlabeled early Maillard products were equal.

The identified glycated tryptic fragments of fibrinogen were collected in Tables S1–S9 (Supplementary Material). These data were obtained for various P:G (protein–glucose) molar ratios. The experiments were repeated several times, applying various combinations of experimental conditions including disulphide bridges reduction, tryptic hydrolysis and chromatographic separation. The majority of modified peptides derived from two chains of fibrinogen: alpha-E and gamma-B. The glycation at P:G 1:370 ratio provided the lowest number of identified peptide – derived Amadori products (4 and 7 in two independent experiments) and the residues K195, K71 from alpha-E chain and K166, K146 from gamma-B chain proved to be the most susceptible to glycation in these conditions (Tables S1 and S2). The increase in molar excess of glucose to 1:500 and 1:1000 resulted in a larger number of detected

Table 2
Reduction of the abundance of the peak of Amadori product containing $^{12}C_6$ glucose in comparisons that containing $^{13}C_6$. These values reflect a glycation inhibition of various lysine residues in fibrinogen caused by HSA.

m/z	*z*	Sequence	AA	Reduction [%]
647,9809	3	[70–84] alpha 1	K71	42
646,0782	4	[191–210] alpha 1	K195, K202	50
707,5666	5	[191–218] alpha 1	K195, K202, K210	38
654,3214	3	[244–258] alpha 1	K249	35
894,7768	3	[89–111] gamma B	K101	55
732,1192	4	[89–113] gamma B	K101, K111	44
1122,0751	2	[135–151] gamma B	K146	44
741,5504	5	[154–185] gamma B	K166, K177	54
660,8205	6	[154–188] gamma B	K166, K177, K185	50

peptide derived Amadori products 18 (Table S4) and 26 respectively (Tables S3 and S6).

The other repetition of analysis of fibrinogen glycated at P:G ratio 1:1000 performed at different experimental conditions (Tables S7, S8 and S9) gave worse sequence coverage – the number of detected glycated peptides was 9, 17 and 7 respectively.

Based on the above data we listed the amino acid residues most susceptible to glycation for each chain, which are presented in Fig. 2. which shows the total number of peptide-derived Amadori products detected in all performed experiments.

The collected results indicated that lysines in chains αE and γB are glycated more efficiently that lysines in chain β. The residues K71, K195, K249 K239, K101, K146, and K166 were found the most frequently in analyzed data set. Interestingly this residues are in agreement with glycation sites indentified in fibrinogen glycated at the lowest P:G ratio (residues K195, K71 from alpha-E chain and K166, K146 from gamma-B chain).

These model experiments provided preliminary information which may be useful for interpretation of *in vivo* fibrinogen glycation results. Most of these data corresponds well with literature reports by Boddi et al. [23]. The preferred glycation sites were the same and in many cases glycated peptides identified in the tryptic digest have the same sequences.

Selected compounds which had a high enough signal intensity were fragmented in LC–MS/MS mode. Using an energy of 22 eV resulted in characteristic neutral losses of 3 water molecules and 1 formaldehyde molecule. These fragments of neutral losses were observed both for [$^{12}C_6$]- and [$^{13}C_6$]-Amadori products. That is presented in a spectrum in Fig. 3. for [$^{12}C_6$]-glycated peptide. LC–MS/MS is an alternative way to our presented method for identification of glycation. Although this experiment did not confirm directly the sequence of peptide it provided a convincing proof that the investigated molecule is a glycation product. The data presented in several papers [24,25] demonstrated that collision dissociation of peptide-derived Amadori products gives a low abundance of peptide backbone fragments and multiple neutral losses of water, formaldehyde, and whole hexose which makes sequencing problematic.

3.2. Differential glycation of fibrinogen

The LC–MS analyses were undertaken for tryptic digests of mixtures of two fibrinogen samples. The first sample contained fibrinogen, glucose with natural isotopic composition and HSA while the second one – fibrinogen and $^{13}C_6$ glucose. Glycation reactions were performed separately but in exactly the same conditions. Before analysis equal volumes of both samples were subjected to reduction of disulphide bridges and tryptic hydrolysis and then mixed. Identified glycated peptides are collected in Table 1. In this experiment glycation was performed in solution, however detected fragments were similar to that observed for solid phase glycation.

The analysis of Table 1 reveals glycation sites which according to data obtained during solid phase reaction were the most susceptible to glycation including residues K71, K195, K249 K239, K101, K146, and K166.

A repetition of analysis gave comparable results, confirming the majority of detected glycation sites.

The obtained data allowed for direct comparison of reactivity toward glucose of selected amino groups in a fibrinogen molecule. The isotopic distribution as well as extracted ion chromatograms presented in the Fig. 4 show clearly that abundance of the peak of Amadori product containing $^{12}C_6$ glucose is significantly lower as compared to that containing $^{13}C_6$ glucose. Since all experimental condition were identical, this difference is a result of interaction of fibrinogen with HSA. Data illustrating mentioned effect are presented in Table 2. In all cases addition of HSA results in decrease of glycation efficiency. The biggest observed effect is almost 60% but inhibition of fibrinogen glycation strongly depends on the position of amino acid residue.

Bhonsle [15] reported that albumin competes for glycation with other plasma proteins and HbA1c. This conclusion is supported by the *in vitro* experiment based on differential depletion of albumin. Low levels of albumin are associated with increased glycation of these proteins. One of the examined proteins was fibrinogen. Two modified sites detected herein are consistent with Bhonsle's data: K114 from gamma chain and K239 from beta chain.

According to our study the effect of albumin on glycation of certain lysine residues in fibrinogen is relatively strong and can not be explained by competition between proteins for glucose. The concentration of glucose (0.5 M) is high in comparison to the concentration of albumin, even taking into consideration multiple amino groups in HSA molecule. Result of the experiment should be rather attributed to protection of certain amino acid moieties in fibrinogen by interacting HSA molecule. This hypothesis finds support in the dependence of protecting effect of albumin on location of the glycated amino acid moiety in fibrinogen. There are evidences that albumin may protect certain proteins from denaturation. Such chaperone-like features of bovine serum albumin reported in several papers [26–28] indicate that albumin may interact in that way with proteins.

In this work applied the combination of isotopic labeling with high resolution mass spectrometry to identify the glycation sites in fibrinogen molecule. We also investigated the influence of albumin on glycation of fibrinogen. Our results correspond with the first study [15] that showed the importance of albumin levels in the regulation of glycation of plasma proteins. However, our approach provided the additional information concerning the HSA influence on glycation of selected amino acid moieties in fibrinogen molecule.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.08.025.

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