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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-