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Identification and comparative quantitation of glycation by stable isotope labeling and LC–MS

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ABSTRACT

Glycation is a common modification of proteins both in vitro and in vivo. To aid identification and comparative quantitation, a method of stable isotope labeling followed by LC–MS analysis was proposed. The samples were reduced using sodium borohydride or sodium borodeuteride. Reduction of the Schiff base between the amine group and the reducing sugars resulted in a molecular weight increase of 2 Da using sodium borohydride or a molecular weight increase of 3 Da using sodium borodeuteride. The molecular weight difference of 1 Da between peptides containing glycated lysine residue reduced using sodium borohydride or sodium borodeuteride was used to identify glycated peptides and to calculate the glycation difference between samples. The method was used to investigate glycation of a recombinant human IgG1 antibody under native and denaturing conditions. The result demonstrated a good correlation between glycation propensity of lysine residues and their solvent exposure levels.

1. Introduction

Glycation is a non-enzymatic reaction between reducing sugars and protein N-terminal amino group and the side chain of lysine residues. The initial product between the amine group and the aldehyde group is a Schiff base, which undergoes a slow step of Amadori rearrangement to form a stable ketoamine, which can further form various advanced glycation end-products (AGEs). Glycation of recombinant proteins can occur during cell culture where sugars are used as major nutrients [1–4] and storage [5–8] where sugars are used as excipients to stabilize proteins. Depending on the sites of glycation, it may impact protein structure, stability and functions [9–12]. In vivo glycation has been implicated in several disease mechanisms [13–15].

Many methods have been used historically to detect glycation [16–18]. One commonly used method, boronate affinity chromatography separates glycated proteins from unglycated proteins thus determining the overall level of glycation [19,20]. Boronate affinity resins have also been used to enrich glycated peptides [21–24]. The total amount of glycation can also be determined by amino acid analysis after reduction of the Schiff base using sodium borohydride [25]. Liquid chromatography and mass spectrometry (LC–MS) has played an essential role in glycation analysis. Glycation can be readily determined by measuring the molecular weights of proteins by mass spectrometry [1,2,4,6–8,18,26–30]. In addition to the traditional approach of fraction collection and Edman degradation [4,31], LC–MS has also become a common approach to determine site-specific glycation [1,2,6–8,18,21,26,29]. In general, glycated peptides can be detected by searching peptides with the molecular weight increase of 162 Da based on the theoretical molecular weights of the peptides. More specifically, glycated peptides have been selectively detected using neutral loss experiments [32]. The sites of glycation have been determined by various fragmentation techniques. Fragment ions caused by loss of water are dominant in the collision induced disassociation (CID) MS/MS spectra of glycated peptides [18,21,26,29,32,33]. However, fragmentation using a relatively higher level of collision energy can result in fragmentation of the peptide backbone with the sugar moiety still attached to the lysine residue [32]. The glycation site can also be determined by CID after reduction using sodium borohydride [29] or fragmentation using electron-transfer dissociation (ETD) [21,22,33].

Methods using ^{13}C-labeled glucose have been developed to aid the determination of protein glycation using mass spectrometry based methods. Incubation of proteins with endogenous glycation with ^{13}C-glucose can generate unique isotopic patterns with molecular weight difference of 6 Da. This pattern enables both identification and relative quantitation of protein glycation in vivo [24].

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Similarly, incubation of proteins with an equal molar mixture of ^{12}C and ^{13}C glucose helps to identify in vitro glycation sites [34]. Based on the assumption that the same residues would be glycated under in vitro condition and in cell culture conditions, the sites of glycation identified using the unique isotopic patterns can help identify glycation from cell culture that is usually present at very low levels at each site [30].

In the current study, an alternative method of differential stable isotope labeling was established to aid identification and comparative quantitation of glycated peptides. Samples of interest were reduced using either sodium borohydride or sodium borodeuteride, which resulted in molecular weight increases of 2 Da or 3 Da, respectively. The 1 Da molecular weight difference was used to identify peptides with glycation. The molecular weight difference of 1 Da was also used to calculate the relative percentage of the level of glycation when the samples were analyzed as a 1:1 mixture.

2. Materials and methods

2.1. Materials

A recombinant monoclonal antibody was expressed in a Chinese hamster ovary (CHO) cell line and purified at Alexion (Cheshire, CT). Human adrenocorticotropic hormone fragment 18–39, acetonitrile, ammonium bicarbonate, dithiothreitol, formic acid, glucose, iodoacetic acid, sodium borohydride, sodium borodeuteride and trifluoroacetic acid (TFA) were purchased from Sigma (St. Louis, MO). Trypsin was purchased from Worthington Biochemical Corp. (Lakewood, NJ).

2.2. In vitro glycation

The antibody was incubated with glucose at two different conditions. First, the recombinant monoclonal antibody at a concentration of 1 mg/mL in phosphate buffer, pH 8.0, was incubated with 0.1 M glucose at 37 °C for 14 days. This sample was used to establish the method. Second, the antibody at a final concentration of 5 mg/mL in phosphate buffer, pH 8.0, was incubated under either native condition or in the presence of 6 M guanidine hydrochloride. When incubated under native conditions, glucose concentrations of 0.01 M, 0.02 M, 0.05 M or 0.1 M were used. When incubated in the presence of 6 M guanidine hydrochloride, glucose concentrations of 0.1 M, 0.2 M, 0.3 M, 0.4 M, 0.5 M or 1 M were used. The peptide ACTH 18–39 was co-incubated in all the samples at a final concentration of 20 μg/mL and used as an internal control to determine the level of glycation. The samples were incubated at 37 °C for 10 days.

2.3. Trypsin digestion

The samples at a final concentration of 1 mg/mL were denatured and reduced in the presence of 6 M guanidine hydrochloride and 10 mM DTT in 20 mM Tris buffer, pH 8.0, at 37 °C for 30 min. Then the samples were alkylated using iodoacetic acid at a final concentration of 30 mM at 37 °C for another 30 min. The samples were then buffer-exchanged into 20 mM ammonium bicarbonate using NAP-5 columns (GE Healthcare, Piscataway, NJ). Digestion was carried out using trypsin at a final enzyme to protein ratio 1:10 at 37 °C for 4 h.

2.4. Reduction

The samples after digestion were reduced using either sodium borohydride or sodium borodeuteride. Stock solutions of 400 mM of sodium borohydride and sodium borodeuteride were prepared in 0.02 M sodium hydroxide immediately prior to use. The stock solution was added to the samples to a final concentration of 20 mM. Reduction was carried out at room temperature for 30 min. The samples were then acidified using formic acid and stored at -20 °C until LC–MS analysis.

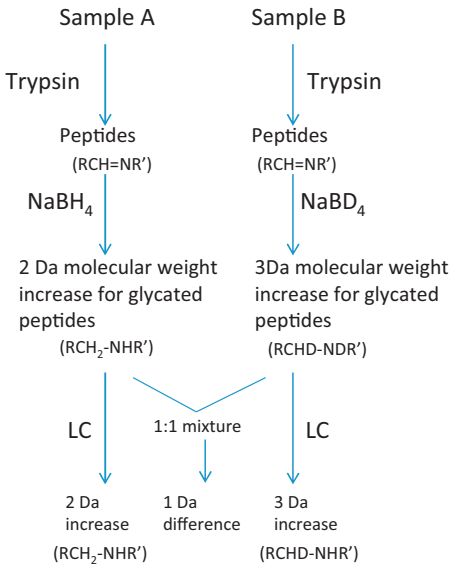

Fig. 1. Workflow of the newly proposed method.

2.5. LC–MS

An Agilent 1100 series HPLC and an LC/MSD mass spectrometer (Santa Clara, CA) were used to analyze protein digests. Each sample of approximately 10 μg was injected into a Zorbax C18 column (1.0 × 150 mm, Agilent) at 95% mobile phase A (0.02% TFA, 0.08% formic acid) and 5% mobile phase B (0.02% TFA and 0.08% formic acid in acetonitrile). After 5 min, mobile phase B was increased to 35% within 100 min to elute and introduce the peptides into the mass spectrometer. The column was then washed using 95% mobile phase B and equilibrated using 5% mobile phase B before the next injection. Throughout the analysis, the flow-rate was set at 50 μL/min and the column temperature was set at 60 °C. The mass spectrometer was run at positive mode with an m/z from 200 to 3000.

3. Results and discussion

3.1. Establishment of the method

The workflow of this newly proposed method is shown in Fig. 1. In brief, the samples were digested separately and then reduced using either sodium borohydride or sodium borodeuteride. Reduction of the Schiff base between the lysine residue and the reducing sugars resulted in a molecular weight increase of 2 Da if reduction was carried out using sodium borohydride, 1 Da from the incorporation of a hydrogen atom from sodium borohydride to the carbon atom of the Schiff base and 1 Da from the incorporation of another hydrogen atom from water to the nitrogen atom of the Schiff base. The molecular weight increase is 3 Da if reduction was carried out using sodium borodeuteride, 2 Da from the incorporation of a deuteride atom from sodium borodeuteride to the carbon atom of the Schiff base and 1 Da from the incorporation of a hydrogen atom from water to the nitrogen atom of the Schiff base. The molecular weight difference of 1 Da between the samples reduced with either sodium borohydride or sodium borodeuteride was used to