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Conformational changes of recombinant monoclonal antibodies by limited proteolytic digestion, stable isotope labeling, and liquid chromatography–mass spectrometry

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ABSTRACT

Limited proteolytic digestion is a method with a long history that has been used to study protein domain structures and conformational changes. A method of combining limited proteolytic digestion, stable isotope labeling, and mass spectrometry was established in the current study to investigate protein conformational changes. Recombinant monoclonal antibodies with or without the conserved oligosaccharides, and with or without oxidation of the conserved methionine residues, were used to test the newly proposed method. All of the samples were digested in ammonium bicarbonate buffer prepared in normal water. The oxidized deglycosylated sample was also digested in ammonium bicarbonate buffer prepared in ^{18}O-labeled water. The sample from the digestion in ^{18}O–water was spiked into each sample digested in normal water. Each mixed sample was subsequently analyzed by liquid chromatography–mass spectrometry (LC–MS). The molecular weight differences between the peptides digested in normal water versus ^{18}O–water were used to differentiate peaks from the samples. The relative peak intensities of peptides with or without the C-terminal incorporation of ^{18}O atoms were used to determine susceptibility of different samples to trypsin and chymotrypsin. The results demonstrated that the method was capable of detecting local conformational changes of the recombinant monoclonal antibodies caused by deglycosylation and oxidation.

Limited proteolytic digestion is a classical method used to study the domain structures and conformational changes of proteins [1,2]. Proteases attack regions of proteins that are exposed and flexible and leave the well-defined compact structures intact [3]. Multiple-domain proteins are typically linked by exposed and flexible regions; therefore, limited proteolytic digestion can be used to define the domain structures [4–10]. Limited proteolytic digestion has also been used to detect protein conformational changes because those changes most likely result in differences in the degradation patterns from limited digestion. The capability of detecting conformational changes by limited digestion has been demonstrated in studies of many different proteins with conformational changes caused by binding to ligands [4–7,11,12], protein–protein interaction [10,12,13], incubation at increased temperature [5], exposure to extreme pH [13,14], and post-translational modifications [15].

The domain structure and conformational changes of IgG molecules, which are a class of molecules with multiple domain structures, have been well studied using various enzymes. Papain and pepsin are two of the widely used enzymes that cleave IgG into different domains. During recent years, IdeS, which cleaves IgG in the lower hinge region to generate F(ab')$_2$ and Fc, has become an important tool to characterize IgG at the subunit level [16–20]. Limited Lys-C digestion has been used to cleave IgG1 in the hinge region to generate fragments similar to those from papain digestion to aid in the localization of posttranslational modifications [21]. To generate even smaller IgG fragments that still maintain some biological functions, limited digestion has been explored extensively [22–25]. Limited proteolytic digestion has also been used for the

Abbreviations used: DSC, differential scanning calorimetry; LC–MS, liquid chromatography–mass spectrometry; TFA, trifluoroacetic acid; DTT, dithiothreitol; UPLC, ultra-performance liquid chromatography; N-mAb, native antibody; D-mAb, deglycosylated antibody; NOxi-mAb, native antibody after oxidation; DOxi-mAb, deglycosylated antibody after oxidation.

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study of conformational changes of various IgG molecules. Conformational changes and decreased stability of IgG molecules without the conserved oligosaccharides have been detected by limited digestion [15,26,27]. Such changes are also observed by differential scanning calorimetry (DSC) [28,29]. Conformational changes and decreased stability of IgG molecules caused by oxidation of two susceptible methionine residues in the Fc region of IgG molecules have been detected by limited proteolytic digestion [15] and further supported by DSC and nuclear magnetic resonance (NMR) studies [30]. Conformational changes caused by low pH incubation were also evidenced by the observation of differences in the digestion pattern from limited digestion of IgG at different pH values [31].

The cleavage pattern and progression of limited digestion are commonly analyzed by a decrease in enzymatic activity [4,9], the disappearance of intact proteins and appearance of fragments by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) [4–7,9–12,14], and chromatography profiles [9–14]. Identification of the cleavage sites have been achieved using Western blot [9], N-terminal sequencing [5–7,10,11,14], amino acid analysis [14], and mass spectrometry [11–14]. A procedure of limited proteolysis and selected reaction monitoring (SRM) by liquid chromatography–mass spectrometry (LC–MS) has been established to evaluate protein conformational difference in complicated biological sample matrix [32]. In this procedure, differential tryptic peptide intensities of the same sample that is either completely digested by trypsin or digested under a limited digestion condition followed by a complete digestion were used to localize protein conformational changes.

In the current study, a new procedure that combines limited proteolytic digestion, proteolytic catalyzed incorporation of ^{18}O-labeled water, and LC–MS was established. A recombinant monoclonal antibody was deglycosylated. Aliquots of the native and deglycosylated antibodies were incubated with hydrogen peroxide to oxidize the susceptible methionine residues. All of the samples were digested by limited digestion in ammonium bicarbonate prepared in normal water. The oxidized deglycosylated sample was also digested in ammonium bicarbonate prepared in ^{18}O–water. Equal volumes of the sample digested in ^{18}O–water were mixed with the samples digested in normal water. Populations of peptides from each sample representing digestion in either normal water or ^{18}O–water were detected by LC–MS. The relative intensity of the peaks was used to determine the susceptibility of each sample to limited trypsin or chymotrypsin digestion.

Materials and methods

Materials

The recombinant monoclonal IgG1 antibody was expressed in a Chinese hamster ovary (CHO) cell line and purified at Alexion (Cheshire, CT, USA). Acetonitrile, ammonium bicarbonate, formic acid, hydrogen peroxide (30%), iodoacetic acid, trifluoroacetic acid (TFA), dithiothreitol (DTT), and ^{18}O–water (97% purity) were purchased from Sigma (St. Louis, MO, USA). Trypsin was purchased from Promega (Madison, WI, USA). Chymotrypsin, Lys-C, and *N*-octylglucoside were purchased from Roche (Indianapolis, IN, USA). PNGaseF was purchased from Prozyme (Hayward, CA, USA).

Deglycosylation and oxidation

The recombinant monoclonal antibody was diluted to 5 mg/ml using phosphate-buffered saline (PBS) at pH 7.4. *N*-Octylglucoside was included in the sample dilution at a final concentration of 1% to facilitate deglycosylation. Deglycosylation was carried out by the addition of PNGaseF to the diluted sample to a final ratio of 1 μl enzyme/100 μg antibody and overnight incubation at 37 °C. Aliquots of the deglycosylated antibody and of the antibody without deglycosylation were then incubated with a final concentration of 5% hydrogen peroxide at room temperature for 2 h to oxidize the susceptible methionine residues in the Fc region. All of the samples were buffer exchanged into the final buffer of 50 mM ammonium bicarbonate using Zeba columns (Thermo Scientific, Rockford, IL, USA). An aliquot of the oxidized deglycosylated sample was buffer exchanged into 50 mM ammonium bicarbonate buffer prepared using ^{18}O–water. Buffer exchange was performed twice to ensure complete buffer exchange.

Limited digestion

The concentrations of the buffer-exchanged samples were determined based on ultraviolet (UV) absorption at 280 nm using the extinction coefficient. The samples were then diluted into 1 mg/ml using the respective buffers. The concentrations were measured and adjusted, if necessary, to ensure that all of the samples were at the same concentration.

Limited digestion was performed using trypsin and chymotrypsin. The enzymes were reconstituted using normal water to a final concentration of 2 μg/μl. To each 100 μg of sample, 2.5 μg of each enzyme was added. After incubation at 37 °C for 30 min, digestion was quenched by the addition of 2 μl formic acid to each sample. Digestion of each sample in normal water was performed in triplicate. Digestion of the oxidized deglycosylated sample in ^{18}O–water was carried out in one experiment.

LC–MS verification of deglycosylation and oxidation

Each sample of 20 μl at a concentration of approximately 1 mg/ml was digested using Lys-C at a final 1:200 Lys-C/antibody ratio. After incubation at room temperature for 15 min, the samples were reduced by the addition of DTT at a final concentration of 20 mM and then incubated at room temperature for 5 min. The reactions were quenched by adding formic acid to lower the sample pH.

A maXis 4G mass spectrometer (Bruker, Billerica, MA, USA), an ultra-performance liquid chromatography (UPLC) system (Waters, Milford, MA, USA), and a Proto 300 C4 column (150 × 1.0 mm, Higgins Analytical, Mountain View, CA, USA) were used to measure the molecular weights of the antibody samples after Lys-C digestion. Approximately 10 μg of each sample was injected into the column at 90% mobile phase A (0.1% TFA and 5% acetonitrile in water) and 10% mobile phase B (0.1% TFA in acetonitrile). After 2 min, the percentage of mobile phase B was increased to 80% within 26 min and remained at 80% for 2 min. The column was equilibrated with 10% 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 in positive mode with the following settings: a scan range of m/z 900 to 5500, gas temperature of 220 °C, drying gas of 10 L/min, nebulizer at 2 psig, and voltage at 120 eV.

LC–MS analysis of samples after limited digestion

Tryptic peptides were analyzed using the same mass spectrometer and the same UPLC system with a Proto 200 C18 column (150 × 1.0 mm, Higgins Analytical). The samples were loaded at 95% mobile phase A (0.1% TFA in water) and 5% mobile phase B (0.1% TFA in acetonitrile). After 5 min, mobile phase B was increased to 35% within 80 min and then to 80% within 20 min. The column was then washed and equilibrated. The column was heated at 60 °C, and the flow rate was set at 50 μl/min. The mass spectrometer was tuned