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Structural characterization of expressed monoclonal antibodies by single sample mass spectral analysis after IdeS proteolysis

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ABSTRACT

Simple and rapid methods for analysis of monoclonal antibody structure and post-translational modifications are increasingly needed due to the explosion of therapeutic monoclonal antibodies and monoclonal antibody applications. Mass spectral analysis is a powerful method for characterizing monoclonal antibodies. Recent discovery and commercialization of the Immunoglobulin G-degrading enzyme of Streptococcus pyogene (IdeS protease) has facilitated and improved the generation of antibody fragments of suitable size to allow characterization of the structure of the entire antibody molecule via analysis of just a few fragments. In this study, we coupled IdeS fragmentation and simultaneous reduction and alkylation of the resultant fragments using tributylphosphine and iodoacetamide to prepare samples in about 2 h. Following simple introduction of a single, unseparated mixture of alkylated fragments into a mass spectrometer, detailed structural information is obtained, covering the entire antibody molecule. The large majority of the glycoforms present on the single, conserved N-linked glycosylation site of the heavy chain is elucidated, although some of the very low abundance glycoforms are not determined by this protocol. The ease, simplicity, speed, and power of this method make it attractive for analysis of the developmental stages and production batches of therapeutic monoclonal antibodies.

1. Introduction

Monoclonal antibodies (mAbs) have become increasingly important therapeutics agents, and now constitute a substantial percentage of therapeutics in medical use, drugs newly approved by the FDA, and agents in human clinical trials. Unlike small molecule drugs, large therapeutic mAbs are more complicated in terms of their production, purification, and quality control parameters. Because therapeutic mAbs are typically recombinant proteins produced in stably transfected cell lines, there are multiple variations in post-translational modifications on a given monoclonal antibody. Thus, there is an ever-increasing need to efficiently characterize antibodies for post-translational modifications, and to subsequently evaluate the *in vivo* ramifications of these varying post-translation modifications. Our laboratory has developed a humanized mAb that has high affinity for cocaine and specificity for cocaine over its inactive metabolites [1]. Furthermore, this recombinant mAb protein can now be produced in Chinese hamster ovary (CHO) cells in gram quantities [2]. This humanized anti-cocaine mAb is currently in an advanced stage of pre-clinical development as a potential therapeutic for the prevention of relapse in cocaine abusers. The next major development milestone is the selection of the best producing cell line to establish a Master Cell Bank. However, this mAb, like most IgG_1 isotypes is glycosylated [2], and has a number of post-translational modifications, including glycosylation, which can lead to structural, and possibly functional, heterogeneity.

Antibody glycosylation is an especially important post-translational modification that may increase antibody solubility and stability, as well as serving to possibly decrease their tendency to aggregate, which are all important properties for any therapeutic

Abbreviations: mAb, monoclonal antibody; h2E2, humanized monoclonal antibody against cocaine; IdeS protease, Immunoglobulin G-degrading enzyme of Streptococcus pyogene; TBP, tributylphosphine; IAM, iodoacetamide; Tris(2-carboxyethyl)phosphine hydrochloride, TCEP; DTT, dithiothreitol; Guanidine hydrochloride, Gu-HCl; TBS, tris-buffered saline; HPLC, high performance liquid chromatography; ESI-Tof MS, electrospray ionization time-of-flight mass spectrometry; CHO, Chinese hamster ovary cell.

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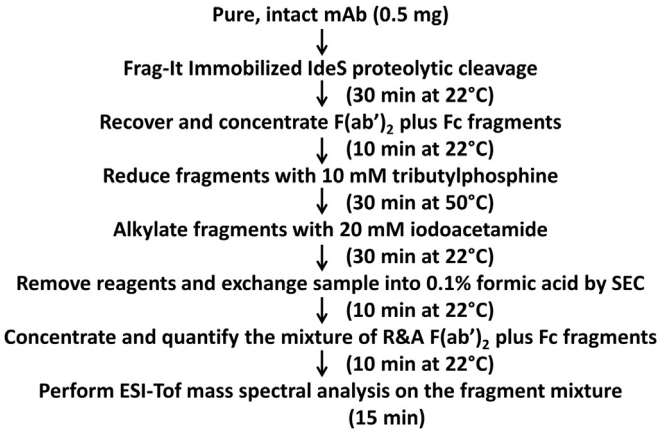

Fig. 1. Flow chart of the mass spectral mAb sample preparation method.

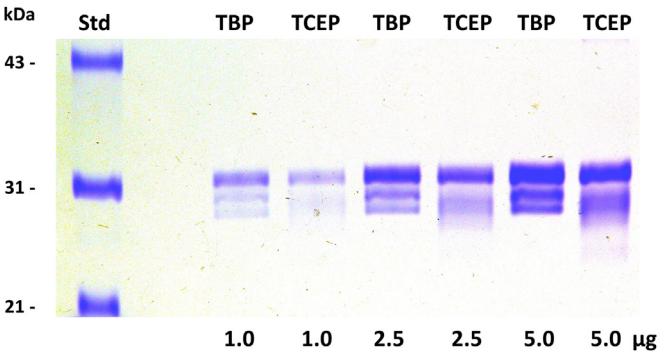

Fig. 2. Comparison of cell line 85 h2E2 mAb samples prepared following the protocol outlined in Fig. 1 using TBP or TCEP as the reductant. Cell line 85 mAb samples were prepared for mass spectral analyses, either using the protocol delineated in Fig. 1, or using a modification of the Fig. 1 protocol with TCEP replacing TBP as the reductant. Multiple aliquots of the resulting samples were analyzed by 10% SDS-PAGE, without DTT reduction prior to electrophoresis. The gel was subsequently stained with Coomassie blue.

protein. Glycosylation can be variable even within production batches and in cell lines with high levels of mAb expression. Slight variations in cell culture medium components and methods can result in changes in expression yields, as well as differential glycoform distributions for mammalian cell expressed antibodies, which could have functional and therapeutic consequences. Thus, methods to quickly, easily, inexpensively, and accurately elucidate the variation and extent of glycosylation and other post-translational modifications located anywhere on the antibody molecule are critical. In this study, we describe such a method to prepare reduced and alkylated fragments of our recombinant anti-cocaine mAb from three cell lines that have high expression levels of the mAb. All post-translational modifications resulting in a mass change located anywhere on the antibody molecule can be assessed using the preparation method described in this work, followed by a relatively simple and inexpensive form of mass spectral analysis. The results obtained can be easily visually inspected and compared in a single reconstructed mass spectrum covering a mass range of only a few thousand daltons. This novel and efficient combination of well-established methods should also be applicable to a broad range of recombinant mAb proteins and should be useful as a routine screening tool to quickly evaluate different cell lines producing the same cloned mAb or different production batches using the same cell line. Because the major glycoforms can be rapidly and quantitatively evaluated, the effects of the use of different media and other expression components on these important post-translational modifications can be quickly evaluated, facilitating the optimization of therapeutic mAb production.

2. Materials and methods

2.1. Materials

Frag-It kits containing immobilized IdeS protease (Immunoglobulin G-degrading enzyme of *Streptococcus pyogene*) columns designed for cleavage of up to 0.5 mg mAb were purchased from Genovis (cat. # A2-FR2-025). Tributylphosphine (TBP) (a 200 mM solution in N-methyl-2-pyrrolidinone (cat. # T-7567)) and iodoacetamide (IAM, cat. # I-6125) were from Sigma. Tris(2-carboxyethyl)phosphine hydrochloride (TCEP, cat # 20,490) was obtained from Thermo Scientific Pierce. The Vivaspin 500 µL concentrators (cat. # 28-9322-25) were from GE Healthcare. Guanidine hydrochloride (Gu-HCl, cat. # A1449) was from AppliChem. To separate TBP and IAM from the fragmented antibody after reduction and alkylation, Sephadex G-25-80 size exclusion beads were from Sigma, and disposable columns were from BioRad (Econo-Pac 14 cm–high 1.5 × 12 cm polypropylene columns, cat. # 7321010EDU). Tris base, NaCl, and concentrated formic acid, sequencing grade, were from Fisher Scientific.

2.2. Methods

Typically, 0.5 mg of the purified h2Es anti-cocaine mAb Fab was processed for analysis, since this is the maximum amount suggested for IdeS proteolysis using the immobilized enzyme on the small Frag-It spin column. Thus, after washing the column and diluting the sample with tris-buffered saline, pH = 7.3 (TBS), a Frag-It spin column was used to cleave 0.25 ml of intact (0.5 mg) mAb into F(ab')$_2$ plus Fc fragments by incubation with end-over-end rotation of the capped column for 30 min at 22 °C. The hydrolyzed mAb sample was recovered by a 1 min, approximately 300 g spin, followed by a wash with 0.25 ml TBS, and subsequent spin. After pooling both samples, the 0.5 ml F(ab')$_2$ plus Fc fragments were concentrated to less than 0.15 ml using a Vivaspin 500 10 kDa MWCO filter.

Reduction and alkylation of the F(ab')$_2$ plus Fc fragments (without separation) was done in a total volume of 0.5 ml, by diluting the concentrated, digested mAb sample in 4 M Gu-HCl, 100 mM Tris-Cl, pH = 8.0, containing 10 mM TBP. Reduction was carried out for 30 min at 50 °C, followed by cooling to 22 °C, and addition of IAM to a final concentration of 20 mM. Alkylation was carried out for 30 min at 22 °C in the dark. Immediately following the alkylation, all excess reagents were removed and the buffer was exchanged at 22 °C for 0.1% formic acid by application of the sample to a 4–5 ml G-25 column, poured in water, and then equilibrated with 0.1% formic acid just before use. The absorbance of each 0.5 ml fraction (280 nm) was determined and the fractions containing non-separated F(ab')$_2$ plus Fc fragments were pooled and concentrated as above. After concentrating to a volume of approximately 30–50 µL, a 5 µL aliquot was diluted 20 fold for determination of yield and protein concentration (typically 3–5 mg/ml, calculated using the $E_{1\%}$ for this mAb of 1.0 mg/ml = 1.463 absorbance at 280 nm). The remainder of the sample was kept at 4 °C. SDS-PAGE analysis of about 0.5–1.0 µL of each preparation was performed, with or without additional reduction with DTT, to check for the expected three fragments of approximately 23–26 kDa.

The resultant samples were analyzed by electrospray ionization time-of-flight mass spectrometry (ESI-Tof MS). Samples were typically diluted to between 150 and 400 ng/uL by transferring 1 µL of the concentrated sample in 0.1% formic acid into 19 µL of 50%

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