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Identification and quantification of Fc fusion peptibody degradations by limited proteolysis method

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ABSTRACT

An Fc fusion protein expressed in *Escherichia coli* contains Met1 and Asp2 residues at the N terminus and an active peptide attached to the C terminus of the Fc region. Due to the unique amino acid sequence of Fc, many commonly used proteolysis methods have severe drawbacks for characterizing degradations of Met1 and Asp2 residues. A novel method has been developed to effectively characterize the degradations by employing a limited endoproteinase Glu-C digestion. The limited digestion generates a dimeric peptide of $(Met1\text{-}Glu14)_2$ due to specific cleavage at the residue Glu14 of the N terminus. This peptide together with its degraded products, including Met1 oxidation and Asp2 isomerization, can be identified and quantified by liquid chromatography–tandem mass spectrometry (LC–MS/MS). The optimization of digestion procedure and linearity of quantification are also described. This approach was successfully used in a photostability study to assess the product stability of an Fc fusion peptibody.

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IgG1 Fc fusion proteins have emerged as potential therapeutics and are under extensive clinical development in the biopharmaceutical industry due to advantages over small molecules (increased specificity) and monoclonal antibodies (small size) [1,2]. Peptide Fc fusion protein usually contains target peptide attached to an immunoglobulin Fc region through its N-terminal or C-terminal direction [3]. The peptide Fc fusion protein described in this work is an active Fc fusion peptibody attached at the C terminus via a flexible linker. The linker is composed of a multiple glycine repeat and is designed to provide sufficient mobility to the peptide moieties necessary for competitive ligand binding.

Chemical degradation plays an important role in protein instability and presents challenges during drug product development, storage, and transportation [4–6]. Fc fusion peptibody tends to exhibit more chemical instability compared with monoclonal antibodies (mAbs)[1] due to their unique structural properties [7]. The methionine (Met or M) and aspartic acid (Asp or D) residues at the N terminus of Fc are prone to oxidation (Met) and isomerization (Asp). Therefore, appropriate methods are required to characterize these degradations and assess the stability during product and process development.

Although peptide mapping in conjunction with tandem mass spectrometry (MS/MS) analysis has been widely used to characterize covalent modifications for larger proteins, many peptide mapping methods are not well suited to monitor the N-terminal Met and Asp degradations of Fc fusion peptibody [8–10]. The common peptide mapping methods, such as trypsin and Lys-C proteolysis, have severe drawbacks even in detecting the peptides containing Met1 and Asp2. This is because trypsin or Lys-C cleavages generate a three-residue MDK peptide, which is usually not retained on most reversed-phase high-performance liquid chromatography (HPLC) columns due to its low hydrophobicity.

In this work, we report a simple and effective approach to achieve easy identification and accurate quantification of chemical modifications associated with the N-terminal Met and Asp residues. The approach is based on the structural flexibility of the N-terminal region and the use of a limited proteolysis by endoproteinase Glu-C. Under a nonreducing condition, Glu-C protease specifically cleaves the Fc fusion peptibody at the C terminus of Glu14 residue, resulting in a dimeric peptide composed of $(Met1\text{-}Glu14)_2$. The $(Met1\text{-}Glu14)_2$ peptide and the remaining portion of the Fc fusion peptibody are then analyzed by LC–MS/MS. Both digestion procedure and LC–MS/MS methods were optimized, and the linear-

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[1] *Abbreviations used:* mAb, monoclonal antibody; MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid; UV, ultraviolet; ESI, electrospray ionization; TOF, time-of-flight; UPLC, ultra-performance liquid chromatography.

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ity of quantification was verified as well. This approach was successfully applied to other Fc fusion peptibodies.

Materials and methods

Materials

Five Fc fusion peptibodies were produced at Amgen (Thousand Oaks, CA, USA). The Fc fusion peptibody samples were statically thawed at room temperature and stored at 4 °C prior to digestion. Endoproteinase Glu-C was purchased from Roche (Indianapolis, IN, USA). HPLC-grade solvents were obtained from Burdick & Jackson (Muskegon, MI, USA). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL, USA). Urea was purchased from J.T. Baker (Phillipsburg, NJ, USA).

The bulk peptibody materials were formulated with a buffer containing 10 mM glutamic acid (pH 5.0), 8% sucrose, and 0.01% polysorbate 20. The starting materials had a purity of greater than 97% based on size exclusion HPLC except where noted. All chemicals used were analytical grade or higher.

Endoproteinase Glu-C digestion

The Fc fusion peptibody was subjected to limited proteolysis using endoproteinase Glu-C. The protein in the formulation buffer was diluted to 1 mg/ml with deionized water and incubated in the presence of endoproteinase Glu-C at an enzyme-to-substrate mass ratio of 1:20 at 25 °C for 4 to 16 h. The solution was quenched with 5% TFA solution followed by HPLC/ultraviolet (UV)/MS analysis.

Reversed-phase HPLC separation

The fragment peptides were separated by reversed-phase HPLC using an Agilent 1100 HPLC equipped with a diode array detector, autosampler, flow cell and temperature-controlled column compartment (Agilent Technologies, Palo Alto, CA, USA). A Varian Metachem Polaris C18 column (250 × 2.1 mm i.d.) packed with a 3-μm diameter, 300-Å pore size C18 resin (Varian, Torrance, CA, USA) was used for the separations. The solvents were as follows: mobile phase A, 0.1% TFA in water; mobile phase B, 0.1% TFA in 95% acetonitrile. The column was equilibrated with 2% solvent B. The two-stage gradient was from 2% to 20% B within 40 min, followed by a second gradient from 20% to 90% B within 1 min. A flush step was performed with 90% B for 9 min, and the column was equilibrated with 2% B for 20 min. The column temperature was maintained at 45 °C. The absorbance of the eluent was monitored at a 215-nm wavelength.

MS analysis of the separated peptides

The HPLC system was directly coupled to an Agilent MSD ion trap mass spectrometer (Agilent Technologies) equipped with an electrospray ionization (ESI) source. The spray voltage was 4.5 kV, and the capillary temperature was 350 °C. The fragmentation mass spectra were obtained using ion trap collision energies of 35%. Each full scan mass spectrum was followed by a zoom scan and then an MS/MS scan of the most intense peak from the full scan. The dynamic exclusion feature was enabled: repeat counts, 2; repeat duration, 0.3 min; exclusion duration, 5 min; exclusion mass width, 2 Da.

Separation of Glu-C digested Fc fusion peptibody

The Glu-C digested peptibody sample was analyzed using the Agilent 1100 HPLC system mentioned above. Separation was performed on a Zorbax SB CN column (150 × 1.0 mm, 3.5 μm, 300 Å) that was heated at 35 °C to enhance separation. Mobile phase A consisted of 0.1% formic acid in water, and mobile phase B consisted of 80% *n*-propanol, 10% acetonitrile, 10% H_2O, and 0.1% formic acid. The column was equilibrated at 10% mobile phase B. Next, 1 min after sample injection, the concentration of mobile phase B was increased to 28% over 1 min, followed by a linear gradient of 28% to 35% B over 33 min. The column was reequilibrated by ramping up mobile phase B to 100% over 1 min, followed by 5 min at 100% B, then down to 10% B over 1 min, followed by 5 min at 10% B. UV absorption was monitored at a 215-nm wavelength.

MS analysis of intact protein

MS was performed on a Micromass LCT mass spectrometer. The ESI–TOF (time-of-flight) mass spectrometer was tuned for maximal MS signal. Positive V mode with resolution of 6000 at *m*/*z* 400 was used to analyze the intact Fc fusion peptibody. Capillary and sample cone voltage was set to 3400 and 100 V, respectively. Ion guide was set to 100 V in order to obtain good intact protein MS signal. An *m*/*z* range of 400 to 3999 and a scan time of 1 s were used during data acquisition. The instrument was calibrated using NaCIS prior to analysis. The MaxEnt1 incorporated part of MassLynx software was used for mass deconvolution.

Results and discussion

Limited proteolysis with endoproteinase Glu-C

A limited proteolysis procedure was applied to protein characterization for posttranslational and chemical modifications of therapeutic proteins such as antibodies and Fc fusion peptibodies. The approach often provides easy sample preparation and quick detection of specific residue degradations, and it simplifies the analysis and characterization of therapeutic Fc fusion peptibodies and antibodies [11–14]. Therefore, it is advantageous over many conventional peptide mapping approaches.

Met1 and Asp2 residues at the N terminus of the Fc region can undergo oxidation and isomerization, respectively. To characterize these degradations, peptide mapping is often the method of choice. To achieve efficient digestion, trypsin and Lys-C are typically used and protein often must undergo denaturation and reduction steps [15]. However, for analyzing degradations related to Met1 and Asp2 residues, digestion with either trypsin or Lys-C results in a

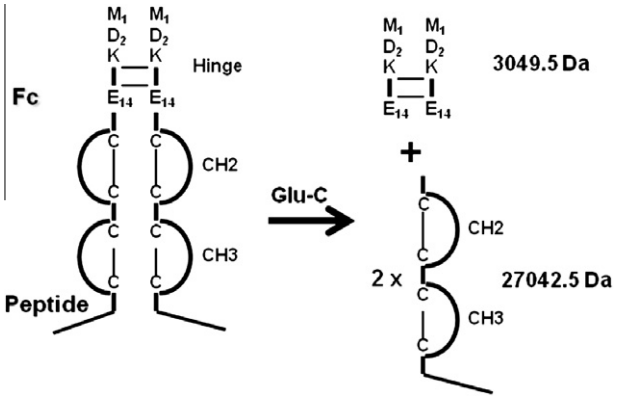

Fig.1. Scheme of the Fc fusion peptibody indicated by limited proteolysis with endoproteinase Glu-C. The cleavage at residue Glu14 (E14) of the N-terminal region produces a dimer peptide of $(Met1\text{-}Glu14)_2$ and the remaining fragment of the Fc fusion peptibody with masses of 3049.5 and 27042.5 Da, respectively.