Deciphering Trisulfide Modification in a Monoclonal Antibody Using a Comprehensive Mass Spectrometry Approach

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Monoclonal antibody (mAb)

Monoclonal antibodies represent an important class of biopharmaceuticals. Immunoglobulin G (IgG), the most common type of mAb therapeutic, is a large multidomain disulfide-bridged protein susceptible to structural and molecular heterogeneity caused by post-translational modifications (PTMs). To ensure product quality and patient safety, it is essential for analysts to characterize and closely monitor these modifications.

Trisulfide modification

Trisulfides, resulting from insertion of a sulfur atom into a disulfide bond of cysteine residues (Cys-S-S-S-Cys; +32 Da), are infrequently documented PTMs of proteins and present a major analytical challenge. Although reports are limited, trisulfides have been found to be a common modification in natural and recombinant antibodies of all IgG subtypes [1]. Therefore, a multilevel analytical workflow for trisulfide characterization is needed.

Comprehensive LC/MS approach

Here we present a comprehensive LC/MS approach to fully characterize trisulfide modifications in a human IgG mAb produced from Chinese Hamster Ovary (CHO) cells. The workflow utilizes top-down, middle-up, and bottom-up approaches to localize and quantify the trisulfide bonds. In addition, we have successfully implemented a simple biochemical approach that efficiently converts the trisulfide bonds back to the native intact disulfides.

Introduction

Experimental

Antibody production

Human IgG1 was produced from Chinese Hamster Ovary (CHO) cells using two different methods: 1) a roller bottle culture by an outside vendor (SDIX), 2) a hollow fiber bioreactor by the Agilent R&D lab (Agilent CrossLab, Santa Clara). The mAb was affinity purified using protein A.

Roller Bottle
(fed-batch culture; prolonged exposure in media)

Hollow Fiber Bioreactor
(continuous process; media continuously refreshed)

Intact mAb analysis

Samples were analyzed on a dual ESI Agilent 6224 TOF LC/MS with an Agilent PLRP-S reversed phase column. MassHunter BioConfirm software was used to deconvolute the intact mass spectra.

Subunit analysis

The mAb was digested with IdeS and Igde enzymes to generate various fragments. Dithiothreitol (DTT) was used to reduce the mAb for heavy chain (HC) and light chain (LC) analysis.

Peptide mapping

The mAb was digested with trypsin and/or Lys-C under non-reducing conditions. Digested samples were desalted and separated using an Agilent AdvanceBio Peptide Mapping column on an Agilent 1290 LC system coupled on-line with an Agilent 6550 iFunnel Q-TOF.
Results and Discussion

After reduction, neither HC or LC contained the modification. A reduced Fab sample was then prepared but similarly, after reduction, the side peak was no longer detected. These results suggest disulfide heterogeneity of the mAb.

The mAb was successfully produced and intact mass analysis matched the predicted mass (149,982 Da), but samples from the roller bottle culture contained a small side peak ~30-40 Da higher than the predicted masses for all glycoforms.

**Subunit analysis (digestion)**

Analysis of subunits generated using IdeS enzyme showed that the modification was on the Fab region of the mAb and not the Fc region.

**Subunit analysis (reduction)**

After reduction, neither HC or LC contained the modification. A reduced Fab sample was then prepared but similarly, after reduction, the side peak was no longer detected. These results suggest disulfide heterogeneity of the mAb.

**Side peak not present after DTT reduction**

The side peak was not detected after DTT reduction, indicating that the modification was not due to disulfide bonds.
A multilevel LC/MS workflow for trisulfide identification, localization, and quantification

A simple chemical approach for conversion of trisulfides to native disulfides

Valuable insight for the development of mAb based analytical standard at Agilent

Results and Discussion

Conversion of trisulfide to disulfide at the intact mAb level

Disulfide mapping revealed a +32 Da mass shift on the non-reduced Lys-C digested peptide that contains the HC:LC interchain cysteine linkage on the Fab region. The trisulfide modification of the mAb is restricted to this region and is present at ~2%.

**Trisulfide %**

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<thead>
<tr>
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<th>Interchain (HC:LC)</th>
<th>Interchain (HC:HC)</th>
<th>Intrachain</th>
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<tbody>
<tr>
<td>Trisulfide</td>
<td>1.88%</td>
<td>0%</td>
<td>0%</td>
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**Reference**


Conclusions

- A multilevel LC/MS workflow for trisulfide identification, localization, and quantification
- A simple chemical approach for conversion of trisulfides to native disulfides
- Valuable insight for the development of mAb based analytical standard at Agilent

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