Structural Characterization of IgG2 Disulfide Isoforms Using Cation Exchange Chromatography Coupled to Mass Spectrometry and Peptide Mapping

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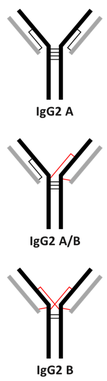
INTRODUCTION

Unlike other IgG subclasses, IgG2 contain 4 cysteines in the hinge region including 2 consecutive ones prone to disulfide bond rearrangements resulting in 3 isoforms of IgG2: A, A/B and B.

This study presents a native Cation EXchange chromatography-Mass Spectrometry (CEX-MS) method using volatile salts to separate IgG2 disulfide isoforms. By combining this approach with a middle-up strategy targeting F(ab')2 fragments, we achieved optimal chromatographic separation and high-quality MS spectra while eliminating Fc-related heterogeneity.

The methodology allowed for the determination of isoform elution order through redox treatment and site-directed mutagenesis, and it enabled the first characterization of an IgG2 mutant designed with a pseudo-isoform B structure to favor agonistic activity.

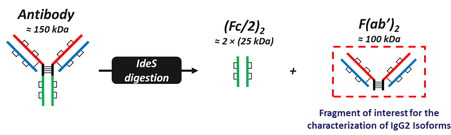
Furthermore, the development of an optimized non-reduced peptide mapping technique, enhanced by an alternative identification criterion: the isotope envelope confidence score, provided a reliable way to identify complex interchain disulfide patterns in the hinge region that conventional methods often fail to resolve.



METHODS

Cation exchange chromatography coupled to mass spectrometry

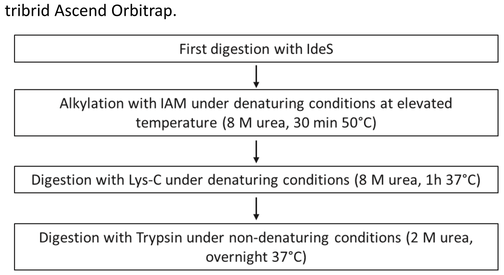
For the characterization of IgG2 isoforms, we used a middle-up analytical strategy. This approach provides superior chromatographic resolution and simplifies the interpretation of mass spectra for antibody fragments. IgG2 are digested with the IdeS enzyme to generate Fc/2 and $F(ab')_2$ fragments. The analysis then focuses on the $F(ab')_2$ region, where disulfide bond rearrangements occur.



CEX-MS was performed with a Proteomix SCX-NP5P 2.1 mm × 250 mm column (Sepax Technologies) and a Vanquish Horizon UPLC system coupled to an Ascend Tribrid Orbitrap mass spectrometer (Thermo Scientific). MS-compatible mobile phases were 25 mM ammonium acetate buffered at pH 5.0 with acetic acid (Buffer A) and 250 mM ammonium acetate at pH 6.8 (Buffer B).

Non-reduced peptide mapping

IgG2 disulfide bonds linkages were characterized by non-reduced peptide mapping using a four-step sample preparation protocol optimized to avoid artefactual disulfide scrambling and limit missed cleavages. LC-MS/MS analysis was performed on a high-resolution tribrid Ascend Orbitrap.



RESULTS

Characterization of IgG2 Reference Monoclonal Antibodies by CEX-MS

The separation of four peaks was observed for the $F(ab)_2$ fragment and, thanks to the direct coupling of CEX with MS, were identified as 4 isomers with a mass error of less than 12 ppm. This result highlights the optimal separation of IgG2 isoforms achieved with CEX-MS, which is equal to or better than reversed-phase chromatography. Furthermore, the use of ammonium acetate in the mobile phases avoids adduct formation, providing high-quality MS spectra.

Based on literature, retention times and relative proportions, we can assume that the isomer 1 corresponds to the isoform B, while isomers 2, 3, and 4 correspond to different structures of isoforms A/B and A.

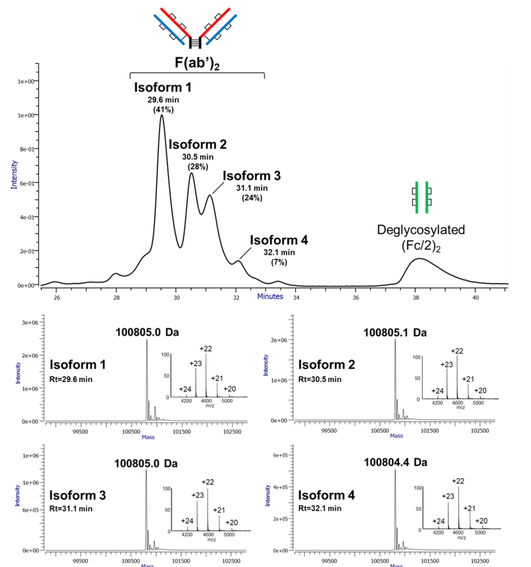
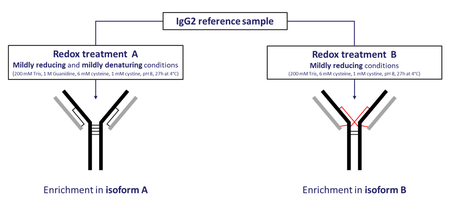


Figure 1. (A) Cation exchange chromatogram of the IgG2 reference mAb after IdeS digestion and deglycosylation (UV detection at 280 nm). (B) Deconvoluted mass spectra of IgG2 F(ab') 2 isomers separated by cation exchange chromatography. Inserts represent the native mass spectra. The theoretical mass of the reference mAb F(ab') 2 fragment is 100,803.8 Da.

Identification of IgG2 Isoforms Using Redox Treatments



To elucidate the elution order of IgG2 isoforms by CEX-MS, we applied redox treatments to the reference IgG2 mab. These treatments are well-documented in the literature for their ability to force the enrichment of specific IgG2 disulfide arrangements. Treatment A, which uses mildly reducing and denaturing conditions, is known to enrich isoform A while treatment B, under mildly reducing conditions only, favors isoform B.

The analysis of these stressed samples must allow us to determine the elution order based on shifts in chromatographic behavior.

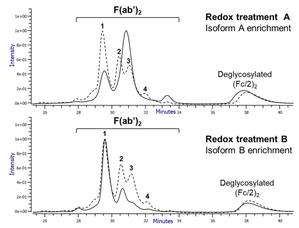
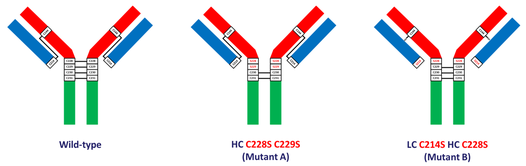


Figure 2. Cation exchange chromatograms of the reference mAb after redox treatments A and B (A and B). The dotted trace represents the reference mAb CEX chromatogram with F(ab') 2 isomers labeled 1 to 4 (UV detection at 280 nm).

For the redox treatment A, we observed a significant decrease in peak 1, which is directly compensated by a sharp increase in peak 3. This tells us that peak 3 corresponds to the isoform A structure.

For the redox treatment B, we see a disappearance of isoforms 2 and 3, while the profile shifts almost entirely in favor of peak 1 suggesting that this peak corresponds to the isoform B.

Identification of IgG2 Isoforms Using Site-Directed Mutagenesis



Site-directed mutagenesis was carried out to create "locked" versions of the antibody that represent the different IgG2 isoform structures. For Mutant A, we performed a double mutation in the heavy chain, replacing the cysteines at positions 228 and 229 with serine residues. This specific modification is designed to block the hinge region in a configuration that mimics Isoform A. For Mutant B we targeted the light chain by mutating Cys214 to Serine, forcing the molecule into a structure that mimics Isoform B.

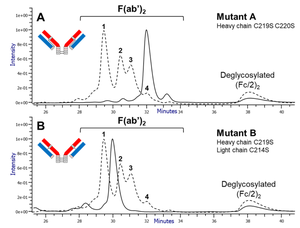


Figure 3. Cation exchange chromatograms of IgG2 mutants A and B (A and B). The dotted trace represents the reference mAb CEX chromatogram with $F(ab')_2$ isomers labeled 1 to 4 (UV detection at 280 nm). Residue numbering is indicated in Eu format.

Mutant A elutes at a significantly higher retention time, matching the later peaks of the reference mAb wich are suspected to correspond to the isoform A. As expected, the mutant B, which mimics the B isoform, elutes much earlier. Its retention time aligns with peak 1 of our reference sample.

Application of the Method to Different IgG2 mAbs

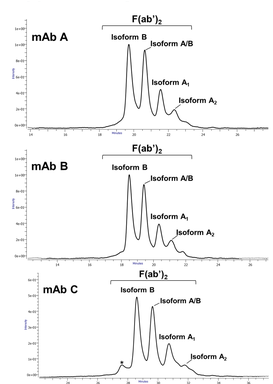
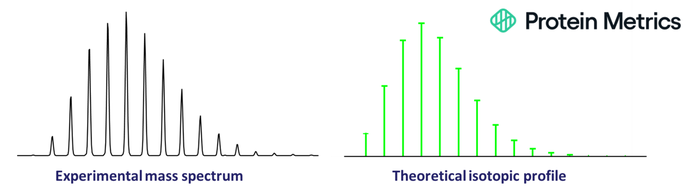


Figure 4. Cation exchange chromatograms of IgG2 F(ab') 2 Article fragments of three different mAbs (UV detection at 280 nm). (*) Proteoform with a mass shift of +18 Da.

The versatility of the method was assessed by analyzing 3 different IgG2 mAbs. All three molecules exhibit the characteristic LC profile of IgG2, with the distinct peaks representing the different IgG2 isoforms. However, some interesting differences were observed depending on Fab variability, such as changes in retention times or improved chromatographic resolution. These results showed that the middle-up CEX-MS approach is not just a "one-off" solution for a single antibody.

Identification of IgG2 Disulfide Bonds Rearrangements by Peptide Mapping



The main challenge for characterizing IgG2 at the peptide level is the high level of structural complexity of hinge-related peptides. With up to six peptides linked by disulfide bonds, MS/MS spectra are highly complex and challenging, if not impossible, to interpret. To overcome this problem, we implemented a robust identification criterion called the **Isotope Envelope Confidence score**, or IEC score. This score performs a comparison between the experimental mass spectrum and the theoretical isotopic profile. It measures the level of concordance between what we detect and what we expect to see based *in silico* generated peptide. This score provides a reliable alternative identification parameter based on high-resolution MS data alone, even when fragmentation patterns are too complex to interpret.

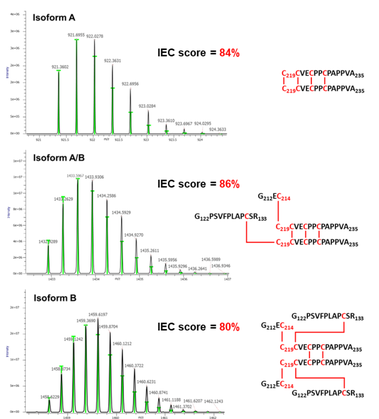


Figure 5. Mass spectra of IgG2 hinge-related peptides of the isoforms A, A/B, and B from wild-type reference mAb. Green bars represent the theoretical isotope envelopes determined by Protein Metrics Byos software for each peptide. Residue numbering is indicated in Eu format.

For data processing, we created an *in silico* inclusion list with every possible hinge-related peptide combination for isoforms A, A/B, and B, considering for up to three missed cleavages.

Then, we applied two stringent parameters for the identification of these peptides based on MS data alone using:

- **Mass accuracy:** Which must be under 3 ppm.
- **The IEC score:** Which must be over 75%.

We achieved IEC scores ranging from 80% to 86% for IgG2 A/B and B isoforms complex hinge clusters.

This method overcomes standard MS/MS limits. Using high-resolution MS and mathematic modeling, we can accurately identify the disulfide bonds for each IgG2 isoform.

CONCLUSION

This study presents the development of the first native CEX-MS method utilizing volatile salts for the effective separation and direct mass spectrometry identification of IgG2 disulfide isoforms. By implementing a middle-up strategy centered on F(ab')2 fragments, we achieved optimal chromatographic resolution and high-quality MS spectra while successfully eliminating interference from Fc-related heterogeneity. By using redox treatments and site-directed mutagenesis, IgG2 isoforms elution order was successfully elucidated. Unlike traditional denaturing techniques, this native approach enabled the detailed characterization of engineered agonist monoclonal antibodies mimicking isoform B, uncovering essential non-covalent subunit interactions.

To further decipher the complex disulfide bond arrangements in the hinge region, we developed an optimized non-reduced peptide mapping workflow that overcomes the limitations of traditional MS/MS interpretation. By introducing an Isotope Envelope Confidence (IEC) score, we achieved confident identification of highly complex interlinked hinge peptides that are otherwise challenging to interpret. This combined approach successfully identified signature peptides for IgG2 isoforms B, A/B, and A, providing a significantly simpler and faster alternative for characterizing interchain patterns compared to existing complex LC-MS/MS methods.

Reference: Anal. Chem. 2025, 97, 17, 9395–9404