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Accurate Sequence Analysis of a Monoclonal Antibody by Top-Down and Middle-Down Orbitrap Mass Spectrometry Applying Multiple Ion Activation Techniques

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Abstract

Targeted top-down (TD) and middle-down (MD) mass spectrometry (MS) offer reduced sample manipulation during protein analysis, limiting the risk of introducing artifactual modifications to better capture sequence information on the proteoforms present. This provides some advantages when characterizing biotherapeutic molecules such as monoclonal antibodies, particularly for the class of biosimilars. Here, we describe the results obtained analyzing a monoclonal IgG1, either in its ~150 kDa intact form or after highly specific digestions yielding ~25 and ~50 kDa subunits, using an Orbitrap mass spectrometer on a liquid chromatography (LC) time scale with fragmentation from ion–photon, ion–ion, and ion–neutral interactions. Ultraviolet photodissociation (UVPD) used a new 213 nm solid-state laser. Alternatively, we applied high-capacity electron-transfer dissociation (ETD HD), alone or in combination with higher energy collisional dissociation (EThcD). Notably, we verify the degree of complementarity of these ion activation methods, with the combination of 213 nm UVPD and ETD HD producing a new record sequence coverage of ~40% for TD MS experiments. The addition of EThcD for the >25 kDa products from MD strategies generated up to 90% of complete sequence information in six LC

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ASSOCIATED CONTENT

Supporting Information

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Extended experimental section, variation of sequence coverage and *p*-score as a function of the fragment mass tolerance and signal-to-noise ratio used for deconvolution of UVPD spectra, TDValidator workflow, tandem mass spectra of intact rituximab fragmented by ETD HD and 213 nm UVPD, graphical fragmentation maps for intact rituximab (IgG1), results of middle-down analysis of rituximab (IgG1) digested with GingisKHAN (without reduction of disulfide bridges) fragmented by ETD, UVPD, and EThcD, results of the characterization of the IdeS-generated ~25 kDa subunit of rituximab fragmented by ETD, UVPD, and EThcD, distribution of ion types produced by 213 nm UVPD, mass accuracy of the four most abundant rituximab glycoforms, and list of transients (i.e., microscans) averaged for different TD MS experiments (PDF)

Notes

The authors declare the following competing financial interest(s): Computational tools described in this work are available as either freeware or via commercialization so a financial conflict of interest is declared and actively managed.

Abstract

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(eBU).¹¹ Sap9 efficiently cleaves IgGs under acidic conditions in about 1 h, reducing the probability of introducing artifacts into the original sample.¹²

However, the analysis of larger subunits or even the whole antibody offers additional information such as the relative order of complementarity determining regions (CDRs) or, in the case of an antibody mixture, the connectivity between light and heavy chains (also known as “chain pairing”) which cannot be inferred from DNA sequencing. The analysis of >10 kDa protein subunits obtained by proteolysis has come to be known as a middle-down (MD) strategy.¹³ Although several proteases can generate large fragments of IgGs, the IgG degrading enzyme from *Streptococcus pyogenes*, IdeS, has become popular.¹⁴ This protease cleaves the heavy chain below the hinge region in <30 min and introduces minimal exogenous modifications.¹⁵ After reduction of disulfide bonds, the IdeS digestion generates three subunits of ~25 kDa each, namely, Lc (light chain), Fd (N-terminal portion of the heavy chain), and Fc/2 (C-terminal portion of the heavy chain). A previous report described the use of electron transfer dissociation (ETD)¹⁶ to fragment and characterize the sequence of the IgG1 adalimumab whose subunits were amenable to high-quality liquid chromatography–tandem mass spectrometry (LC–MS/MS).¹⁷ A single LC–MS/MS run garnered up to 50% sequence coverage on each subunit, while combined results from multiple LC–MS/MS with varied ETD durations increased this value to almost 70%.¹⁷ More recently, Cotham and Brodbelt implemented a similar experimental setup for the characterization of the IgG1 trastuzumab. However, replacing ETD with 193 nm ultraviolet photodissociation (UVPD)¹⁸ yielded even higher sequence coverage, with up to 80% coverage when results of multiple LC–MS/MS runs were considered together.¹⁹ Another study combined results from collision-induced dissociation (CID) and ETD, yielding up to 72% sequence coverage on selected IgG1 subunits using a 21 T Fourier transform MS ion cyclotron resonance (FT-MS ICR) mass spectrometer.²⁰

State-of-the-art analysis of whole IgGs under denaturing conditions, termed top-down (TD) MS,²¹ has been carried out using electron capture dissociation (ECD)²² or the related technique of ETD.²³ Both time-of-flight²⁴ and Fourier transform MS (FT-MS) Orbitrap²⁵ and ion cyclotron resonance (ICR) traps have been employed for fragment ion readout at isotopic resolution.²⁶ In FT-MS experiments, averaging hundreds to thousands of time-domain transients to improve the spectral signal-to-noise ratio (S/N) alongside isolation and simultaneous activation of multiple precursor ions has led to ~30% sequence coverage with ETD or ECD.^{25,26} A new report suggests that the limited sequence coverage obtainable by TD MS is primarily caused by the presence of the disulfide bridges in the intact IgG, which direct fragmentation to disulfide-free regions.²⁷ To improve TD and MD analysis of mAbs with complete molecular specificity and elevate these approaches as valid alternatives to BU in biopharma, further workflow simplification is needed to achieve a robust and reliable platform for characterization, data analysis, and validation.

Here, we combine several recent advances in TD and MD MS of IgGs carried out on a Tribrid Orbitrap mass spectrometer (Orbitrap Fusion Lumos). We analyzed the IgG1 rituximab using both high-capacity electron-transfer dissociation (commercially known as ETD HD)²⁸ and 213nm UVPD,²⁹ along with electron-transfer dissociation–higher energy collisional dissociation (ETHCD).^{27,30} Our analysis considered the intact mAb, as well as the

50 kDa subunits originated by proteolytic digestion with the recently introduced protease GingisKHAN³¹ and the 25 kDa subunits obtained by IdeS proteolysis and disulfide bond reduction. Compared to previous reports, we lowered the number of LC–MS runs required for a comprehensive mAb characterization. Furthermore, we investigated the effects of deconvolution and ion matching parameters on sequence coverage to reduce the number of false positives in matching fragment ions to a candidate sequence. Finally, we used a new software tool, TDValidator, to confirm product ions by matching to theoretical protein sequence-specific isotopic distributions, thereby validating all backbone cleavage events produced from diverse fragmentation methods

EXPERIMENTAL SECTION

Chemicals.

LC–MS grade water and acetonitrile (ACN) were purchased from Fisher Scientific (Rockford, IL, U.S.A.). Formic acid (FA) and trifluoroacetic acid (TFA) ampules were obtained by Thermo Fisher Scientific (Skokie, IL, U.S.A.). Gingipain K protease (GingisKHAN) was purchased from Genovis (Lund, Sweden). IdeS protease was purchased from Promega (Fitchburg, WI, U.S.A.). Guanidine chloride and tris(2-carboxyethyl)phosphine (TCEP) were purchased from Sigma-Aldrich (Milwaukee, WI, U.S.A.).

Sample Preparation.

The therapeutic monoclonal antibody rituximab (Rituxan; Genentech, South San Francisco, CA, U.S.A.) was obtained in its standard formulation buffer in the form commercially available to the general public. For GingisKHAN and IdeS proteolysis, we adapted previously described protocols.^{17,31}

For TD measurements, the antibody was directly loaded onto the column without further purification. In contrast, the ~50 and ~25 kDa subunits obtained by GingisKHAN and IdeS digestion, respectively, were purified via ZipTip desalting (Millipore, St. Charles, MO, U.S.A.) following a previously described protocol¹¹ prior to further analysis. For each measurement, between 0.5 and 1 μ g of IgG or IgG products was used. Additional details are provided in the Supporting Information.

Liquid Chromatography.

For all experiments we applied reversed-phase liquid chromatography (LC) to desalt samples and, when needed, separate the IgG subunits prior to their ionization and injection into the mass spectrometer. The LC setup was based on an Ultimate 3000 chromatographic system (Thermo Fisher Scientific, San Jose, CA), with the column outlet connected online to the ionization source of the mass spectrometer. A binary gradient was used, with the composition of mobile phases as follows: solution A was an aqueous solution of 5% ACN and 0.2% FA (v/v), while solution B was 5% H₂O and 0.2% FA in acetonitrile. Additional details for each experiment can be found in the Supporting Information.

Basic Mass Spectrometry Setup.

All experiments were performed on a Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Scientific) equipped with high-capacity ETD (ETD HD) and 213 nm UVPD options. For performing ETD HD, fluoranthene radical anions were generated by the Townsend discharge ETD source (also known as fETD) positioned in the source region of the instrument.³² For UVPD, the mass spectrometer was equipped with a solid-state Nd:YAG laser whose fifth harmonic was used to generate 213 nm UV photons. Detailed schematics of the UVPD setup were described in a recent publication.³³ All measurements were performed under “intact protein pressure” settings, with the N₂ pressure set at 2 mTorr in the ion routing multipole (i.e., HCD cell).^{27,34} All scans were obtained by averaging 10 time-domain transients (i.e., microscans) prior to signal processing by enhanced Fourier transform (eFT).³⁵ MS² experiments were designed in a targeted fashion, with elution times for species of interest determined through a preliminary LC run performed in MS¹-only mode. All FT-MS spectra were recorded without noise removal using “full profile” mode. Additional details for specific experiments are provided in the Supporting Information.

Data Analysis.

The deconvolution of full MS scans to obtain the masses of IgG subunits was performed either using an in-house generated Excel spreadsheet for isotopically unresolved spectra returning average masses or using the Xtract³⁶ algorithm (Thermo Scientific, included in Qual-Browser) in the case of isotopically resolved spectra. All high-resolution averaged MS² spectra were deconvoluted with Xtract using the following parameters: S/N threshold = 7, remainder 25%, fit factor 60%, maximum charge 40. A list of exact monoisotopic masses was imported into ProSight Lite (available at prosightlite.northwestern.edu)^{37,38} along with the appropriate sequence of rituximab chain/subunit to generate graphical fragmentation maps using a fragment ion tolerance of 10 ppm. Different types of fragment ions were considered for specific ion fragmentation types.

To obtain finalized fragmentation maps, the original list of matched ions was manually curated with the help of a newly released application, TDValidator (available at proteinaceous.-net/product/tdvalidator). TDValidator includes an isotopic fitter algorithm which can match the experimental isotopic clusters from the original .raw spectrum (i.e., non-deconvoluted) using the calculated isotopic clusters of fragment ions for a given polypeptide sequence. The fitter algorithm relies on candidate chemical formulas rather than the averagine model used by Xtract. TDValidator is therefore complementary to “forward approaches” which work from spectrum to neutral mass to match fragment ions with a candidate sequence. TDValidator takes the “reverse approach” of starting from sequence and working back to raw spectral data. The charge state range for fragment ions to be considered is a user-definable parameter. For the analyses reported here, we first considered the list of ions matched using Xtract and ProSight Lite; from there, all the fragment ions identified by Xtract but not matched by the isotopic fitter were manually evaluated and removed if necessary (e.g., if less than three isotopic distributions for a large ion were correctly matched). The graphical fragment maps presented in the figures of this study therefore include only the manually validated fragment ions. The choices for the deconvolution and fragment matching parameters are detailed in the Results and Discussion section.

RESULTS AND DISCUSSION

Software-Assisted Validation of Results Using TDValidator.

As described in the Experimental Section and recapitulated in Figure 1A, assignment of product ions was performed in three steps. First, a traditional spectral deconvolution using Xtract was followed by sequence matching in ProSight Lite. Separately, the newly introduced TDValidator was used for searching unprocessed spectra directly from the .raw file using a customized isotopic fitter (Figure 1B). The results of these two analyses were then compared, and the matched ions not found by TDValidator directly in the spectrum were manually validated and, if needed, excluded from the final list. With regard to the first step, we defined a specific set of parameters for deconvolution and matching. Particularly, we studied the variation of the p -score and the sequence coverage returned by ProSight Lite as a function of the fragment ion tolerance and the S/N threshold used for deconvolution (Figure 2, parts A and B, respectively) to suppress the number of false positive fragment ion assignments in the final sequence coverage (i.e., removing a few true positives). Figure 2A and Figure S-1 both show that the optimal range for fragment tolerance was between 5 and 10 ppm, while reduced values sharply decrease the number of matched fragment ions. Considering that the average mass error for the given data set (based on LC-MS/MS data with limited spectral averaging) is ~5 ppm, we opted for 10 ppm tolerance. As Figure 2B and Figure S-2 show, a plateau was reached in terms of p -score at S/N = 5; we therefore increased the S/N threshold from the standard value of 3 to a higher, more conservative value of 7 (note that S/N = 7 was determined a conservative, appropriate choice by iterating this analysis for all the IgG subunits and applying it also to MS² spectra from standard proteins, data not shown). Figure 1B offers an overview of the main functions of TDValidator and its user interface. For a given polypeptide sequence with specific modifications (i.e., a proteoform),³⁹ and a specific fragmentation type, TDValidator generates in silico theoretical isotopic distributions of ions based on exact atom compositions and matches those to experimental data. When a match is found within specified tolerances, the theoretical distribution is superimposed on the experimental product ion cluster. A vertical bar indicates the position and relative abundance of the theoretical most intense isotopomer. Additional details regarding the use of TDValidator as well as the isotopic fitter algorithm are reported in Figures S-3 and S-4. By generating sequence-specific distributions instead of average distributions, more precise product ion validation on an individual proteoform basis can be achieved, particularly for the cases where the identities of the proteoforms are already known (Figure S-5).

Cleavage Propensities of ETD HD and 213 nm UVPD in Top-Down MS of Rituximab.

The deconvolution of the charge state envelope of intact rituximab, obtained at “medium” resolution in the Orbitrap (i.e., 15 000 resolving power at 200 m/z), recapitulated the population of glycoform variants for this IgG, with the G0F/G1F being the most abundant; importantly, the intact mass determination also indicated the presence of pyroglutamic acid (pGlu) on the N-terminus of both the light and heavy chains and the clipping of the C-terminal lysine of the heavy chain (Table S-1). The presence of these PTMs, previously characterized in rituximab,⁴⁰ was considered for correctly assigning MS²-generated fragment ions. Parts A and B of Figure S-6 show the MS² ETD spectra obtained in two

separate LC runs (averaging 260 microscans/run), allowing electron transfer for 10 or 25 ms. As previously reported for similar experiments, an increase in the ion–ion interaction time leads to the depletion of the charge-reduced species from the spectrum, which are largely present in the 10 ms spectrum due to the 600 m/z -wide isolation window used to improve ETD efficiency.^{25,27} Notably, both 10 and 25 ms spectra took advantage of the high precursor capacity feature implemented in ETD HD, which allows for an approximately 3-fold increase in the number of precursor ions, with proportional improvement in the final S/N of product ions. Therefore, we found it was possible to reduce by 2–6-fold the number of averaged MS² scans compared to previous reports²⁷ and still obtain high-quality spectra (Table S-2). The UVPD spectrum derived from a single LC run is represented in Figure S-6C. This spectrum presents a substantially narrower distribution of product ions (centered on 1250 m/z) compared to any of the ETD counterparts. Similar to the 10 ms ETD spectrum, the UVPD spectrum also shows the clear presence of residual precursors at high mass-to-charge (m/z) values. The most abundant product ions from the UVPD spectrum are more highly charged than ETD-generated equivalents (the average charge of the ions above 10% of the base peak intensity is 9.1 vs 7.0 for UVPD and 10 ms ETD, respectively). This apparent difference in product ion distribution over the m/z space is partially reflected in the regions of intact rituximab that are sequenced by the two ion activation methods. Figure S-7 shows the graphical fragmentation maps for light (top) and heavy (bottom) chains obtained by combining the matched fragment ions from 10 and 25 ms ETD experiments. Similar to previous reports,^{25,27} ETD efficiently sequenced the disulfide-free loops on both chains. In detail, a high degree of coverage was obtained for the areas between the second and third Cys residues on the light chain, the second and third Cys residues at the N-terminus of the heavy chain, and the ninth and tenth Cys residues near the C-terminus of the heavy chain. The sequence coverage reached using ETD corresponded to 21.2% and 25.6% for light and heavy chain, respectively (Table 1). The fragmentation achieved via 213 nm UVPD is summarized by graphical fragmentation maps in Figure S-8. The sequence coverage for the light chain was increased to 31.6%, due to the additional fragment ions matched at both the termini of the sequence. Notably, these new, small ions belonged to all the different types of fragment ions that can be generated by UVPD, without much apparent preference. The majority of matched product ions, however, were from the same region between the second and third Cys residues already characterized by ETD. Conversely, the fragmentation map of the heavy chain showed a dramatic shift in the position of matched N-terminal-containing product ions when compared with the corresponding ETD one: UVPD generated, similarly to ETD, a large number of fragments belonging to the C-terminus (and specifically the aforementioned region between ninth and 10th Cys) but, differently from ETD, did not sequence extensively the N-terminus; instead, it covered the central portion of the chain (approximately, between residues 190 and 250), what structurally is known as the antibody hinge region. Overall, UVPD sequenced 23.4% of the heavy chain, and the high complementarity between ETD and UVPD (Figures S-7 and S-8) gave an overall 41.2% sequence coverage for the larger chain when ETD and UVPD data were combined. The total sequence coverage for the intact IgG, considering both chains and both ion activations, reached 40.2% (Figure S-9).

Characterization of 50 kDa Disulfide-Intact IgG Subunits.

Both the heterodimer Fab and the homodimer Fc obtained by GingisKHAN digestion include inter- and intramolecular disulfide bridges (see Table S-3 for a list of the major digestion products). The results of the fragmentation via ETD (one duration), UVPD (two different pulse numbers), and EThcD (one ETD duration/HCD activation) are summarized in Table 1, and the respective fragmentation maps are displayed in Figures S-10–S-12. The sequence coverage obtained by considering termini-containing product ions (i.e., canonical fragment ions) is typically between 10% and 20%; nevertheless, all the ion activation techniques combined gave 20% overall sequence coverage for all the chains, and reached 36% in the case of the Fc (Figure 3). These results demonstrate the complementarity of ion fragmentation techniques and also suggest a large number of product ions are not accounted by the present analysis as they include parts of two chains linked by intermolecular disulfide bridges, in accordance to what recently reported by Szrenti et al.³¹ The reduced number of matched ions containing the C-terminus of the Fd and Lc chains seems to support this hypothesis, as the intermolecular disulfide bond connecting the two chains is located at the C-terminus of both of them. Branched fragment ions will require more specific consideration in the future, particularly for the ~50 kDa Fab which represents the smallest species that preserves the chain-pairing information that captures combinations of the CDR-variable regions present in intact IgG proteoforms.

Toward Complete Sequencing of Disulfide-Reduced 25 kDa Subunits.

The 25 kDa subunits derived from full disulfide reduction of IdeS digestion products were subjected to fragmentation via ETD, UVPD, and EThcD using a total of six LC–MS² runs. The percent sequence coverage obtained for each single subunit and ion activation type are summarized in Table 1. The Lc and Fc/2 subunits were characterized to a higher degree (with sequence coverage up to total 90%) than the Fd, for which >70% sequence coverage was obtained.^{17,19} Notably, these results were obtained despite that ~5–10% of ETD and up to 15% of UVPD fragment ions automatically matched were removed from the final list in each run after manual inspection using TDValidator. The panels of Figure 4 show the fragmentation maps generated by combining the results of all the ion activations. The fragmentation maps of the three subunits subjected to a single ion activation are shown in Figures S-13–S-15 for ETD, UVPD, and EThcD, respectively. Furthermore, we were able to identify fragment ions retaining N-linked glycans (specifically G0F, G1F, and G2F), and, although the vast majority of N-terminal fragments for Lc and Fd include the pyroGlu modification, also a few fragments containing an N-terminal Gln were observed. Careful inspection of these fragmentation maps reveals the highly complementary nature of the activation techniques: as an example, ETD (three durations) and UVPD (two different laser irradiations) yielded 59.3% and 71.3% sequence coverage for the Fc/2 subunit, respectively. However, while neither of these fragmentation techniques was able to fragment efficiently the last 5–7 residues of the protein termini, EThcD was able to partially characterize them, presumably due to the vibrational activation of primary reaction products from ETD in the HCD cell promoting the formation of smaller protein fragment ions. Furthermore, the two ion activation techniques that result on average in the higher sequence coverage, ETD and UVPD, do not sequence with equal efficiency all the 25 kDa subunits: for instance, Table 1 indicates that UVPD outperforms ETD in sequencing the Fc/2 but returns a lower sequence

coverage than the electron-based fragmentation for the Fd subunit (47.3% vs 38.5% for ETD and UVPD, respectively). The high degree of complementarity between all the three ion activation technologies is summarized for each of the three IgG subunits in the Venn diagrams displayed in the right panels of Figure 4. These diagrams show the matched fragment ions that are shared between ETD, EThcD, and UVPD. Unsurprisingly, this analysis showed a larger number of common identified ions between ETD and EThcD, while UVPD adds the highest number of unique matched ions for each of the subunits. Considering all three ion activation methods, the total number of *unique* matched ions account for 30–43% of the total. This last observation could be explained considering the high number of *a/x*-type ions produced by 213 nm UVPD: these ion types cannot be produced by ETD or EThcD (Figure S-14). Overall, these results underline the importance of applying multiple ion fragmentation methods to obtain the highest achievable sequence coverage.

CONCLUSIONS

These results show that the use of multiple ion activation techniques coupled with state-of-the-art Orbitrap FT-MS enables thorough characterization of biotherapeutics such as monoclonal antibodies: the combination of TD and MD MS not only yields an overall sequence coverage (and PTM mapping) in line with what obtainable by BU or eBU MS strategies, but also produces multiple layers of information about the IgG of interest (e.g., CDR pairing, chain pairing, intact mass) not obtainable after extended proteolysis. Owing to the complementarity between ETD, EThcD, and UVPD, the overall number of LC–MS/MS experiments required for generating high-quality information is low (a total of only 13 LC runs were considered for the main body of this work). To further ease the transition of TD and MD MS technology from “research grade” to “quality control ready” tools, we also introduced more stringent parameters for the standard data analysis workflow (i.e., spectral deconvolution followed by matching of neutral masses) to increase robustness and confidence of final reports. In summary, we envision that the antibody characterization protocol based on TD and MD will be useful not only for the analysis of other classes of biotherapeutics (e.g., antibody–drug conjugates, diverse multivalent mAb conjugates, and monomeric glycoproteins),⁴¹ but also that, in the near future, considering the constant development of new dedicated software⁴² and efficient ion activation technologies,⁴³ TD and MD technologies will serve applications currently performed only by BU MS, such as targeted de novo sequencing.⁴⁴

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) General workflow for the parallel analysis via Xtract/ProSight Lite and TDValidator. (B) Graphic user interface of TDValidator showing a confident match between an experimental (black trace) and a theoretical (red triangles) isotopic cluster for the z_{106}^{10+} fragment ion from MS² by ETD of a subunit liberated from rituximab using the IdeS protease (Fc/2).

Figure 2.

(A) Variation of sequence coverage (expressed as a percentage) and $-\text{LOG}_{10}(\text{p-score})$ as a function of fragment ion tolerance used for sequence matching; the set of observed fragment ions returned by the Xtract algorithm was determined with a signal-to-noise (S/N) threshold of 7:1. (B) Sequence coverage and $-\text{LOG}_{10}(\text{p-score})$ as a function of S/N threshold selected for Xtract, keeping the fragment ion tolerance fixed at ± 5 ppm. For these plots we considered the reduced light chain of rituximab (obtained via IdeS digestion) fragmented by UVPD using five laser pulses.

Figure 3.

Results of middle-down analysis of rituximab digested with GingisKHAN (without reduction of disulfide bonds). (A) Chromatographic separation of the two ~50 kDa IgG subunits produced by proteolysis, which are present in a stoichiometric ratio of 1:2 for Fc and Fab, respectively. Insets show the isotopic distribution of Fc (left) and Fab (right). (B–D) Graphical fragmentation maps obtained by combining ETD, UVPD, and EThcD data for the three subunits: Fd (B) and Lc forming the Fab subunit (C), and the Fc (D). The presence of the N-linked glycan, mapped using diagnostic fragment ions that include the mass of the sugar chains G0F, G1F, and G2F, is indicated by an orange rectangle.

Figure 4.

Results from LC–MS characterization of the IdeS-generated ~25 kDa subunits of rituximab. The left sides of panels A–C show the fragmentation maps of Fd, Lc, and Fc/2, respectively, obtained by summing data from three ETD runs, two UVPD runs, and one EThcD run (total of six LC–MS² runs). The presence of the N-linked glycan, mapped using diagnostic fragment ions which include the mass of the sugar chains G0F, G1F, and G2F, is highlighted by an orange rectangle. For each panel, the right side shows the corresponding Venn diagram of shared/unique matched fragment ions for each of the three ion fragmentation techniques.

Table 1.
Summary of Sequence Coverage of Rituximab Obtained via Top-Down or Middle-Down
Analysis Using Multiple Ion Fragmentation Techniques^a

workflow	species targeted (mass in kDa)	chain	no. of LC runs	sequence coverage (%)			
				ETD	UVPD	EThcD	total
top-down ^b	intact (150)	light	3 ^c	21.2	31.6		38.2
		heavy		25.6	23.4		41.2
middle-down GingisKHAN ^b	Fc (50)	heavy – Fc	4 ^d	17.0	21.5	9.9	36.8
	Fab (50)	heavy – Fd		11.6	13.2	14.7	29.3
		light		10.4	13.8	11.3	25.0
middle-down IdeS ^e	Fc/2 (25)		6 ^f	59.8	61.2	51.7	86.6
	Fd (25)			47.3	38.5	38.5	72.0
	Lc (25)			53.3	50.9	45.3	79.7

^aOne single ion activation was used per LC run. Note that EThcD was not applied to the top-down analysis of intact rituximab.

^bWith all disulfide bridges intact.

^cSumming two ETD runs and one UVPD run.

^dSumming one ETD run, two UVPD runs, and one EThcD run.

^eWith reduced S–S bridges.

^fSumming three ETD runs, two UVPD runs, and one EThcD run.