Cleavage of Intact Monoclonal Antibodies by Cathepsins L and D Studied by Native Mass Spectrometry

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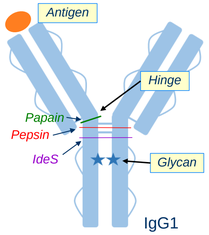
Introduction

Monoclonal antibody (mAb) analysis, unlike proteomics, employs a variety of proteases in order to obtain complete coverage, overlapping peptides, and well-defined subunits. Proteases such as papain, pepsin, and IdeS, cut intact mAbs at specific locations relative to disulfide bonds. These digestion preferences are important for subunit analysis and for mapping disulfide bonds involving cysteine residues near the mAb hinges. (Other disulfide bonds can be effectively mapped with a non-reduced trypsin or pepsin (Liu et al, 2014) digest.)

Here we use high-resolution native mass spectrometry and automated matching of charge-deconvolved masses to investigate the preferred cleavage sites of two lysosomal proteases, cathepsins L and D, on three therapeutic mAbs, including eculizumab (trade name: Soliris), a mixed IgG2 / IgG4 antibody. We show that these two proteases have preferences for particular segments of antibody sequences, for example, the hinge regions of IgG1's. The cleavage specificities of Cathepsin D and L suggest novel ways to check disulfide bonding pattern in IgG's.



Methods – Experimental

We show data from three commercially available therapeutic mAbs, generous gifts from Genmab (Utrecht, The Netherlands):

- Obinutuzumab (Gazyva)
- Rituximab (MabThera)
- Eculizumab (Soliris)

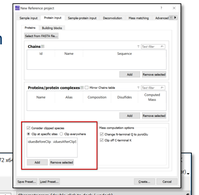
Antibody powder was reconstituted in Milli-Q water. Each mAb was divided in two aliquots, one treated with Cathepsin L and the other with Cathepsin D, both in 1:200 ratio. Samples were then incubated at 37ºC for 2 days.

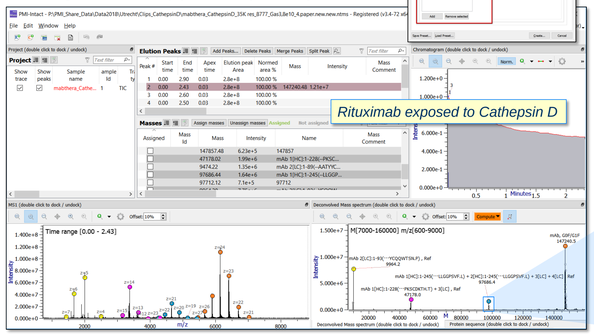
The aqueous mAb samples were buffer exchanged with 150 mM aqueous AMAC (pH 7.5) by centrifugation using a 10 kDa cut-off filter (Merck Millipore). The resulting protein concentration was measured by UV absorbance at 280 nm and adjusted to 2-3 µM prior to native MS analysis. All samples were buffer exchanged to 150 mM AMAC (pH 7.5) prior to native MS measurements. After native MS measurements, PNGase F was added and incubated overnight, and native MS was performed on the deglycosylated samples.

Samples were analyzed on an Exactive Plus Orbitrap EMR (Thermo Fisher Scientific) under native conditions as described previously (Rosati et al, 2014). Source fragmentation and collision energy were varied from 70 to 90 V and resolution was set to either 35,000 or 70,000. Scans covered *m/z* range 500 – 12,000.

Methods – Computational

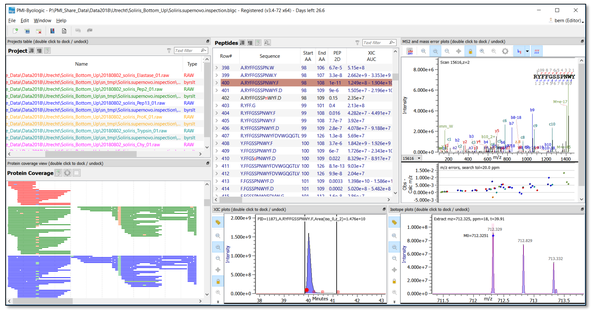
Protein Metrics Intact Mass™ software (Bern et al, 2018) allows the user to define single- and multi-chain proteins, with modifications such as disulfide bonds and pyro-Glu N-terminus. The user can set clipping sites based on flanking residues, for example, D.P or G.G for common hydrolysis sites, or PAPELLG.G for IdeS digestion, or simply allow clips at any site.





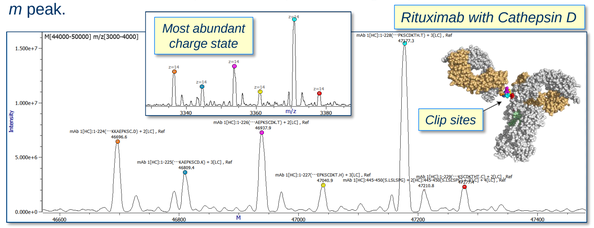
De Novo Sequencing of Eculizumab

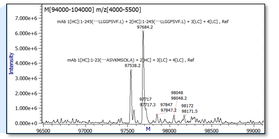
We obtained amino acid sequences for rituximab and obinutuzumab from literature and Web searches, but we could not initially find the sequence for eculizumab HC, without which we could not compute theoretical masses. So we *de novo* sequenced eculizumab, using multiple digestions (trypsin, chymotrypsin, elastase, etc.) and our software Supernovo. We later found the published eculizumab sequence at http://www.freepatentsonline.com/y2017/0073399.html. Our de novo sequence was correct except for one I / L substitution.



Clip Assignments

We initially used full *m/z* and *m* ranges (default parameters) to obtain "survey deconvolutions". We then used a matching tolerance of ±4 Da on average-isotope mass, and allowed clipping between any pair of residues. For greater sensitivity, we focused on particular *m/z* and *m* ranges after studying the survey deconvolution. We find *m* peaks for all possible hinge clips down to ~1% of base *m* peak.





Deconvolution onto 94 – 104 kDa finds a cleavage site at GGPSVF.L, five residues after the IdeS site. The 97,538-Da peak is misidentified; more likely is HC cut at VF.L and at SV.F, but Intact Mass does not look for two-chain clips at two different sites, due to the large number of possibilities.

We compiled lists of cleavages for the three different mAbs and two different proteases. The main finding is that both cathepsins cut at the hinge of rituximab and obinutuzumab, but neither cathepsin cuts off an "arm" of eculizumab. Cathepsin D also cuts below the hinges, most often at GGPSVF.L.

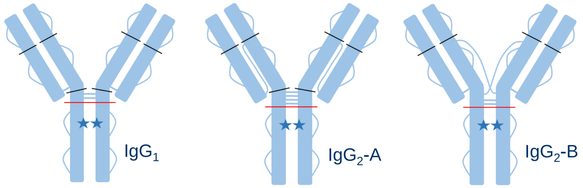
Obinutuzumab exposed to Cathepsin L gives one clip in CDR H3, clips at the end of the variable part of the HC, and a complete sequence at the hinge. Not shown are peaks around 101 kDa, complementary to the hinge peaks. All HC sequences begin with N-terminal pyro-Glu rather than Glu. Finally ? denotes unassigned peaks as intense as some assigned peaks.

Mass	Theoretical	Intensity	Assignment
10866.33	10867.06	8.80E+04	HC 1-99 (···YCARN.V)
14681.96	14682.36	4.55E+04	HC 1-135 (···APSSK.S)
14869.87	14870.55	3.02E+04	HC 1-137 (···SSKST.S)
14957.54	14957.62	2.73E+04	HC 1-138 (···SKSTS.G)
15014.36	15014.68	6.57E+04	HC 1-139 (···KSTSG.G)
22544.58	22545.16	2.27E+04	LC 1-206 (···THQGL.S)
23491.56	23492.22	2.59E+04	HC 1-220 (···KVEPK.S)
23834.75	23835.55	9.00E+04	LC 1-218 (···FNRGE.C)
23904.34		4.75E+04	?
23938.56	23938.69	3.96E+04	LC
24057.36	23957.83	2.82E+05	LC + Cysteinylation
24243.96		4.77E+04	?
47618.71	47619.12	1.81E+06	LC + HC : 1-222 (···EPKSC.D)
47734.36	47734.21	8.32E+05	LC + HC : 1-223 (···PKSCD.K)
47861.76	47862.38	1.92E+06	LC + HC : 1-224 (···KSCDK.T)
47962.73	47963.49	7.09E+05	LC + HC : 1-225 (···SCDKT.H)
48099.86	48100.63	5.31E+06	LC + HC : 1-226 (···CDKTH.T)
48201.09	48201.73	1.00E+06	LC + HC : 1-227 (···DKTHT.C)

Mass	Theoretical	Intensity	Assignment
47619.25	47619.12	3.48E+05	LC + HC : 1-222 (···EPKSC.D)
47734.72	47734.21	2.40E+05	LC + HC : 1-223 (···PKSCD.K)
47861.84	47862.38	4.35E+05	LC + HC : 1-224 (···KSCDK.T)
48101.69	48100.63	8.39E+05	LC + HC : 1-226 (···CDKTH.T)
49616.22	49618.4	4.10E+05	LC + HC : 1-242 (···GGPSV.F)
49765.03	49765.58	7.27E+05	LC + HC : 1-243 (···GPSVF.L)
99381.38		5.71E+06	2LC + HC : 1-245 (...GGPSVF.L) + HC: 1-244 (...LGGPSV.F)
99528.16		1.57E+07	2LC + 2HC : 1-245 (...GGPSVF.L)
99642.25		2.25E+06	2LC + HC : 1-245 (...GGPSVF.L) + HC: 1-246 (...GPSVFL.F)

Obinutuzumab exposed to Cathepsin D gives clips in the HC hinge, along with cuts of the HC separating F(ab')2 from Fc.

Disulfide Bond Patterns of IgG's



Clip sites:
- Cathepsin D only
- Cathepsin L and D

All four cathepsin experiments on the two IgG1's gave nearly complete cleavage of the heavy chain hinge, but neither cathepsin cleaved eculizumab (an IgG2 / IgG4 hybrid), indicating that eculizumab has almost exclusively the IgG2-B disulfide bond pattern, as generally expected for IgG2's with kappa light chains (Liu-May, 2011). All six cathepsin experiments gave some fragmentation near CDRs H3 and L3, that is, between the disulfide-bonded pairs in the mAb "arms".

Altogether the results suggest that digestion with cathepsins followed by native MS offers a method to check much of the disulfide bonding pattern of IgG's.

Conclusions and References

We draw the following conclusions from the work so far:

- Cathepsin L and D offer potentially useful mAb digestion specificity
- High-resolution native MS measures mAb clips with 100x dynamic range.
- Automatic clip matching greatly speeds up analysis, but matches must still be validated manually, because any mass peak has about 10% chance of matching a theoretical clip mass within ±4 Da tolerance.

References

(Liu et al, 2014) Facilitating protein disulfide bond mapping by a combination of pepsin digestion, EThcD, and a dedicated search algorithm SlinkS. Mol Cell Proteomics, 2014, PMID: 24980484

(Rosati et al, 2014) Detailed mass analysis of structural heterogeneity in monoclonal antibodies using native mass spectrometry. Nat. Protoc, 2014, PMID: 24675736

(Bern et al, 2018) Parsimonious charge deconvolution for native mass spectrometry. J Proteome Res, 2018, PMID: 29376659

(Liu-May, 2011) Disulfide bond structures of IgG molecules, mAbs, 2012, PMID: 22327427

Acknowledgments

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