Improved Peptide Mapping of Therapeutic Antibodies using Proteases with Orthogonal Cleavage Specificity

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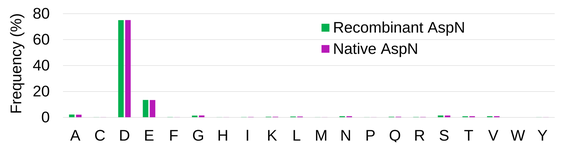
1. Introduction

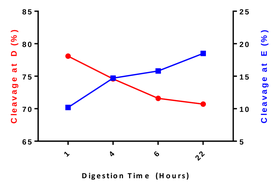
Peptide mapping of therapeutic proteins is typically performed with trypsin. While trypsin is an excellent protease, peptide maps produced with trypsin are not always complete and tryptic peptides may not be suitable to monitor all important quality attributes. In such cases, using alternative proteases with orthogonal sequence coverage can help fill these gaps. In this study we report the characterization of a new recombinant Asp-specific protease, rAsp-N, and utilized the NISTmAb reference IgG to develop protocols for efficient digestion and improved sequence coverage compared to trypsin alone. We further illustrate how sequence coverage can be increased within a single injection by combining digests from both proteases prior to data collection.

2. Recombinant Asp-N is Specific and Efficient

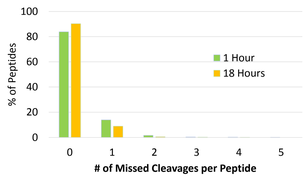
A) Cleavage Specificity



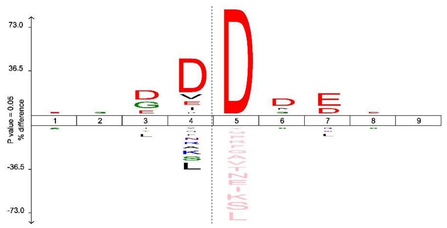
B) Specificity vs. Digest Time



C) Cleavage Efficiency (Asp)



D) IceLogo: Missed-Cleavage Patterns



Yeast extract (Promega V7341) was digested for various times and analyzed by LC-MS/MS. Briefly, Native Asp-N or rAsp-N were added at a 1:50 enzyme:substrate ratio for various times at 37C at pH 8 at a final concentration of 2M urea. Data were searched with Mascot using a "No-enzyme" approach and Scaffold output was further analyzed with Excel to determine the N-terminal amino acid frequency (cleavage specificity) (A & B) as well as efficiency of cleavage (C). IceLogo was used to visualize missed-cleavages (D). The dashed vertical line in the IceLogo plot indicates the missed-cleavage site. Residues above the line are found at higher frequency in peptides with missed D-cleavage.

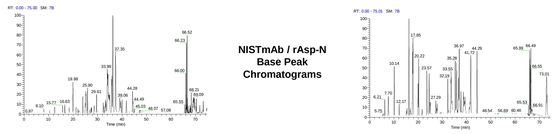
Recombinant Asp-N is highly efficient with specificity similar to the native enzyme.

Missed cleavages generally occur when the cleavage site is preceded by a D

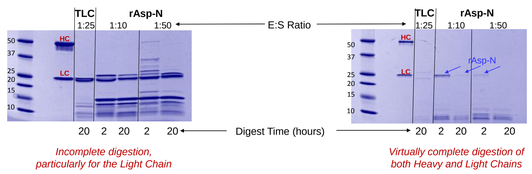
Cleavage at glutamic acid gradually accumulates as digestion times increase

3. NISTmAb Peptide Mapping

A)



B)



C)

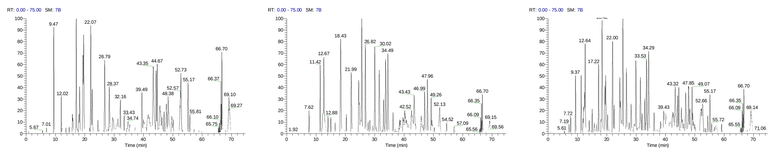
	Denatured with Urea, Diluted to 1.5M					Denatured with GuHCl, Zeba™ Desalted				
	rAsp-N				TLC	rAsp-N				TLC
Time	*2 hrs*		*20 hrs*		*20 hrs*	*2 hrs*		*20 hrs*		*20 hrs*
Ratio	*1:50*	*1:10*	*1:50*	*1:10*	*1:25*	*1:50*	*1:10*	*1:50*	*1:10*	*1:25*
HC coverage (%)	80	83	83	92	86	96	80	92	80	87
LC coverage (%)	51	66	59	74	69	100	99	87	84	88
HC spectra	133	218	164	259	797	665	726	733	585	1306
LC spectra	176	231	176	239	358	611	503	475	294	772

NISTmAb was denatured with 6M GuHCl, reduced and alkylated, then buffer-exchanged into Tris pH 8 using a Zeba™ column. Alternatively, denaturing was done in 7M urea, which was then diluted to 1.5M urea during the digestion step. 20 µg of the reduced and alkylated NISTmAb was digested with 0.4 µg of rAsp-N or 0.8 µg of Trypsin/Lys-C (TLC) mix (Promega) for either 2 or 20 hours at 37°C. ~1 pmole of digested IgG was analyzed on a Q Exactive with a 1hr gradient of 5-40% acetonitrile. Data were analyzed with Mascot and Scaffold. A) Base peak chromatograms of rAsp-N digests of NISTmAb. B) SDS-PAGE analysis of digests. C) Sequence coverage and spectra observed as reported by Scaffold.

Guanidine-HCl is preferred over urea for denaturing NISTmAb prior to digestion

Efficient digestion is achieved at 1:10 for 2 hours or 1:50 from 2 hours to overnight

4. Digests from Multiple Proteases in a Single Injection



	Sequence Coverage		
Digest	**rAsp-N**	**Trypsin/Lys-C**	**Combined**
Search	*Asp-N*	*Trypsin*	*Asp-N & Trypsin*
Heavy Chain	94	88	100
Light Chain	89	80	100

NISTmAb was digested with either rAsp-N (1:50) or Trypsin/Lys-C (1:25) for 20 hours as described in Panel 3 above following the GuHCl denaturing protocol. Digests were terminated by acidification with formic acid (1% final) and then analyzed by LC-MS/MS under two distinct conditions: individually or combined. For individual digests with either rAsp-N or TLC, 1 pmol was injected. For combined digests, equal parts of rAsp-N and Trypsin/Lys-C digests were mixed and then 2 pmole total (1pmol originating from each digest) was injected. All injections were run in duplicate originally on 0-40% acetonitrile gradients. Subsequently injections were run with 0-30% acetonitrile gradients which gave better separation. Data were searched in Mascot and were subsequently further analyzed with Scaffold.

Combining digests from multiple proteases prior to LC-MS/MS increases sequence coverage from a single injection.

5. Asp-N Cleavage at Deamidation Hotspots

The so-called "PENNY" peptide typically refers to the sequence **GFYPSDIAVEWESNGQ*PENNY*K** obtained from trypsin digestion of the heavy chain of IgGs. This peptide contains a hotspot for deamidation, highlighted in red. A similar peptide can be obtained after Asp-N digestion, as seen below:

"N" DIAVEWESNGQPENNYKTTPPVL

Deamidation due to heat, basic pH, etc.

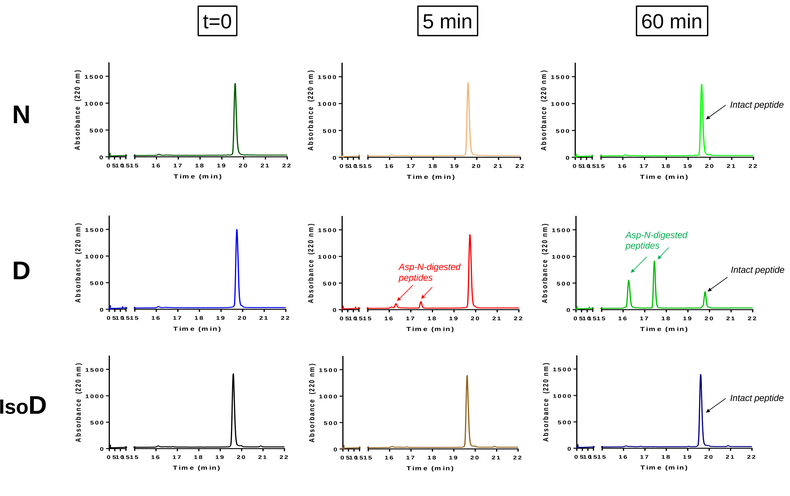
DIAVEWESDGQPENNYKTTPPVL — **"D"**

DIAVEWES(Iso)DGQPENNYKTTPPVL — **"IsoD"**

☑ Should be cleaved by Asp-N

Cleaved by Asp-N?

Incubation of Three Synthetic Asp-N PENNY Peptides with rAsp-N



Synthetic PENNY peptides (Anaspec) were mixed at a 1:250 (rAsp-N:peptide) ratio for various times at 37°C and pH 8. After termination of digestion with TFA, 5 ug of each peptide was injected into an Agilent 1200 HPLC and separated on a C18 column with an acetonitrile gradient.

rAsp-N does not cleave at Isoaspartic acid in the PENNY peptide

6. Conclusions

- rAsp-N is a newly available protease with a strong preference for cleavage at aspartic acid and no cleavage was observed at isoaspartic acid
- Improved format: 10 µg size in a conical vial allows for more consistent resuspension
- Efficient digestion allows for relatively short digestion times ranging from 1-2 hours
- Excellent choice of orthogonal protease for peptide mapping of biotherapeutic proteins
- Combining digests from multiple proteases in a single injection has several advantages:
  - Increases sequence coverage compared to a trypsin digestion alone
  - Reduces demand on instrument time
  - Orthogonal measurements of quality attributes in a single injection
  - Obtain measurements of quality attributes not detected with a trypsin digest

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