Characterization of Therapeutic Antibodies with a Protease that Cleaves after Proline & Alanine

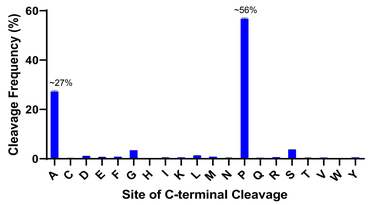
Chris Hosfield[1], Antonio Datola[2], Maura Melchiorre[2], Lucia Colarusso[2], Angelo Palmese[2], Michael Rosenblatt[1] & Marjeta Urh[1]
[1]Promega Corporation, 2800 Woods Hollow Rd, Madison, WI, 53719
[2]EMD Serono, Via Luigi Einaudi, 11 00012 Guidonia Montecelio – Rome, Italy

1. Introduction

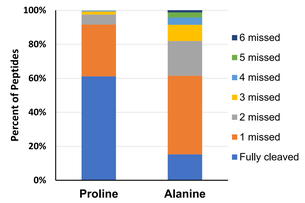
Proteases beyond trypsin are important for protein characterization by mass spectrometry since they help increase sequence coverage and identify post-translational modifications. Like trypsin, the most commonly-used alternative proteases also cleave at charged residues thus there is a need for proteases that cleave at unique sites in the proteome. Here we describe the characterization of ProAlanase, a protease which preferentially cleaves proteins on the C-terminal side of proline and alanine residues. Digestion with ProAlanase is optimal with short durations of 1-2 hours at acidic pH (~1.5-2.0) which suppresses introduction of sample preparation artifacts and minimizes nonspecific digestion. The enzyme digested IgG successfully under both reducing and non-reducing conditions which facilitated characterization of primary structure coverage, PTMs and disulfide bonds.

2. ProAlanase Cleaves C-terminal to Proline & Alanine

ProAlanase Cleavage Specificity



ProAlanase Digestion Efficiency

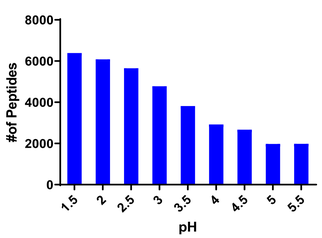


Human K562 extract was digested with ProAlanase at pH 1.5 for 2 hours at 37°C at a 1:100 enzyme:substrate (E:S) ratio. Data were searched with Byonic with no enzyme specified.

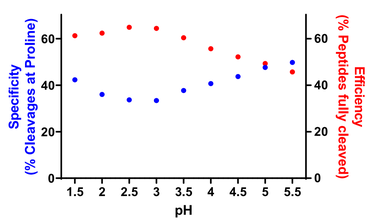
- ProAlanase cleaves proteins primarily C-terminal to proline
- ProAlanase also cleaves C-terminal to alanine, albeit with lower efficiency

3. ProAlanase is Optimal Under Strongly Acidic Conditions

Peptide Identifications



Specificity and Efficiency

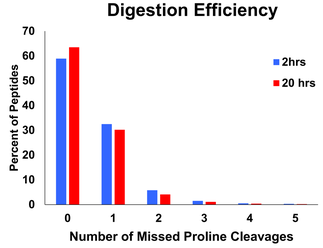


Human K562 extract was digested with ProAlanase at various pH values for 2 hours at 37°C at a 1:50 E:S ratio.

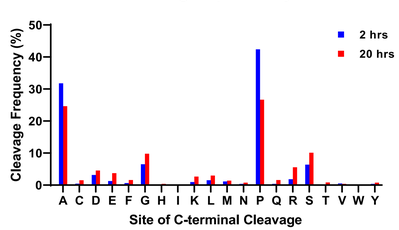
- Results vary somewhat depending on the digestion pH
- pH 1.5 produced the best combination of specificity and peptide identifications

4. ProAlanase is Optimal with Short Digestion Times

Digestion Efficiency



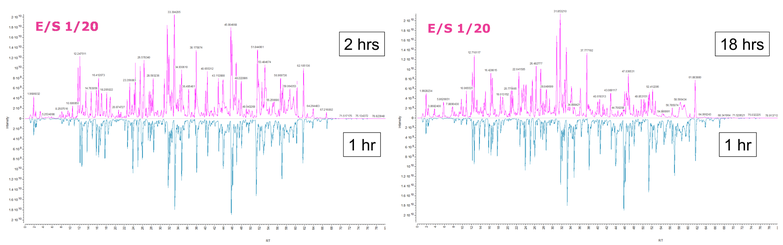
Cleavage Specificity



Human K562 extract was digested with ProAlanase at pH 1.5 for 2 hours or overnight at 37°C at a 1:50 E:S ratio.

- Most proline residues available to be digested are cleaved within 2 hours or less
- Longer digests increase efficiency slightly but result in reduced specificity

5. Optimization of IgG Digestion with ProAlanase



IgG was treated with maleimide to block free cysteines, followed by reduction and alkylation prior to digestion with ProAlanase. Digestions were performed at pH 2.0 and 37°C, at 1:50 (not shown) or 1:20 enzyme:substrate ratios either for 1, 2 or 18 hours. Data from IgG digests were collected on a Thermo Lumos Orbitrap and data were analyzed with Expressionist Refiner MS (Genedata).

- Shorter digestion times (1-2 hours) are preferable and produce comparable chromatograms.
- No undigested protein was observed even after 1 hour
- Longer digestion times (18 hours) leads to some nonspecific cleavages (Gly, Ser, Asp, Lys, Arg)

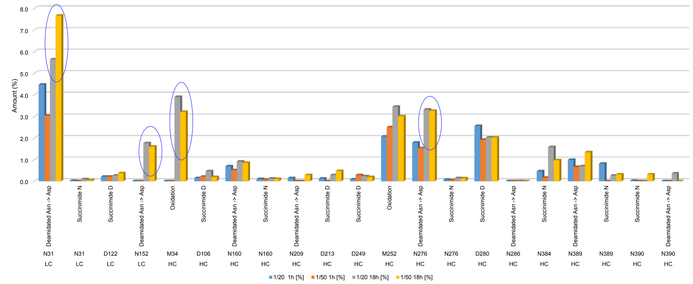
6. Primary Sequence Coverage of IgG

	E/S 1:50		E/S 1:20	
Digest Time	**LC**	**HC**	**LC**	**HC**
1 hour	100	89	100	88
2 hours	89	89	95	88
18 hours	80	86	73	80

IgG was treated with maleimide to block free cysteines, followed by reduction and alkylation prior to digestion with ProAlanase at pH 2.0 and 37°C, at 1:50 or 1:20 enzyme:substrate ratios either for 1, 2 or 18 hours. Data from IgG digests were collected on a Thermo Lumos Orbitrap and data were analyzed with Expressionist Refiner MS (Genedata) in Pro/Ala specific searches (shown above) or non-specific searches (discussed below). Values in the table represent percent amino acid sequence coverage of light chain (LC) or heavy chain (LC).

- Highest sequence coverage is observed with shorter digestion times with 1 hour being optimal.
- Nonspecific database searches can readily produce 100% sequence coverage of both LC and HC.

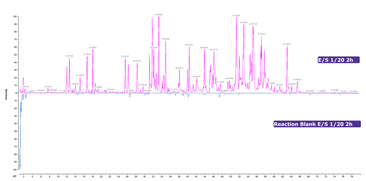
7. PTM Analysis



- ProAlanase is useful for analysis of PTMs, and similar and consistent levels of PTMs are observed during short digests either at 1:20 or 1:50 E:S ratio. Optimization of E:S ratio is recommended.
- Long digestion (18 hrs) at acidic pH resulted in apparent increase in some PTMs (blue ovals)
- Overall, similar levels of PTMs are shown with ProAlanase compared to Trypsin (not shown)

8. Disulfide Bond Analysis

Non-Reduced Peptide Map



Selected Disulfide Bridged Peptides

Digest Time	Peptide	Intensity
1 hr	(P)EDIATYYCQQGNTFP(Y)=(A)SVGDRVTITCRASQDISNYLAWYQQKP(G)	5.E+10
2 hr		7.E+10
18 hr		6.E+10
1 hr	(P)CSRSTSESTA(A)=(A)ALGCLVKDYFP(E)	1.E+09
2 hr		1.E+09
18 hr		1.E+08
1 hr	(A)VYYCA(S)=(A)SVKVSCKASGYTFFSFWMHWVRQAP(G)	9.E+08
2 hr		1.E+09
18 hr		6.E+10
1 hr	(P)CSRSTSESTAA(L)=(A)LGCLVKDYFP(E)	3.E+08
2 hr		3.E+08
16 hr		1.E+07

IgG was digested with ProAlanase under non-reducing conditions after denaturing with 6M GuHCl. Digestions were performed at pH 2.0 and 37°C, at 1:50 (not shown) or 1:20 E:S ratios either for 1, 2 or 18 hours. Data from IgG digests were collected on a Thermo Lumos Orbitrap and data were analyzed with Expressionist Refiner MS (Genedata).

- ProAlanase successfully digests non-reduced IgG and allows for the assignment of most disulfide bridges.
- Acidic digestion conditions are favorable for disulfide analysis and no evidence of disulfide shuffling was seen.
- Minimal autoproteolysis of ProAlanase is observed as seen in the reaction blank with no substrate.
- Longer digestion times lead to increased nonspecific cleavage as seen in reduced peptide maps.
- Optimal conditions for analysis of this molecule were: E/S 1/20 for 1-2 hrs at 37°C.

9. Conclusions

- ProAlanase is a new mass spec grade protease useful in a variety of applications including proteomic analysis & characterization of therapeutic proteins.
- Proteomic analysis of K562 extracts illustrates ProAlanase cleaves primarily C-terminal to Proline (and Alanine to a lesser extent) with optimal activity at acidic pH with 1-2 hour digestion time. Longer digests lead to reduced specificity.
- Primary sequence coverage & PTM analysis of IgGs:
  - Short (1-2 hour) digestion times were optimal for high sequence coverage and PTM analysis.
  - Widening search parameters to account for nonspecific cleavage further increased sequence coverage to 100% for both heavy and light chains.
  - The level of detected PTMs observed with ProAlanase digestion at pH 2 are mostly are similar to the level observed with Trypsin at pH 7.5.
  - Some PTMs appear to be induced during 18 hour digests therefore shorter digests are recommended.
- Disulfide bridge analysis of IgGs:
  - ProAlanase efficiently digests non-reduced IgG molecules at pH 2.
  - Many disulfide bonds have been confirmed particularly those the hinge region.
  - No evidence of disulfide reshuffling was observed as expected at acidic pH.

Corresponding author: chris.hosfield@promega.com