A Recombinant Asp-Specific Protease for Bottom-up Mass Spectrometry Workflows

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1. Introduction

Bottom-up mass spectrometry workflows typically utilize trypsin to digest proteins into peptides suitable for LC-MS/MS analysis. While trypsin is an excellent protease, alternative proteases are useful for numerous applications including increasing protein sequence coverage and identifying post-translational modifications. LysC, another commonly used protease, is also robust and specific, but has specificity similar to trypsin. Site-specific proteases with orthogonal specificity such as GluC and AspN are useful but can suffer from relatively poor digestion performance. Here we report the expression, purification and characterization of a protease which displays both high cleavage efficiency and a strong preference for cleavage N-terminal to aspartic acid. This new recombinant protease should have broad utility for bottom-up LC-MS/MS workflows.

2. Expression and Purification

- Cloned from *Stenotrophomonas maltophilia*
- Expressed in *E coli*.
- Purified to >95% purity
- Contains a C-terminal His tag.

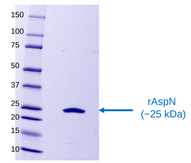
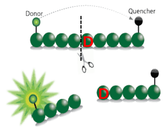


Fig. 1. SDS-PAGE Purity Gel

Recombinant AspN was successfully expressed and purified to >95%

3. Enzyme Activity Assay

- Enzyme activity is measured by FRET using a synthetic peptide substrate
- *S. maltophilia* AspN is a putative Zn-metalloprotease
- Used FRET assay to determine optimal pH for activity and effect of EDTA



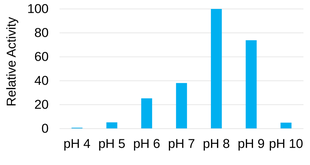


Fig. 2A. Effect of pH on Enzymatic Activity of rAspN

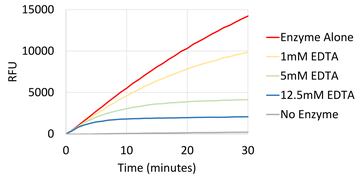


Fig. 2B. FRET Activity Assay and Effect of EDTA

Recombinant AspN from *S. maltophilia* (10 µg, Promega CS1990B01) was reconstituted in 50 µL of nanopure water. A) To measure activity, 10 µM AspN FRET substrate (Sigma A9971) was incubated at 37°C with 1 ng of rAspN in various buffers. B) To measure inhibition, increasing amounts of EDTA were added to the substrate in a 50 mM Tris pH 8 buffer. Reactions were initiated by addition of enzyme and fluorescence was monitored at 430 nm after excitation at 320 nm.

Recombinant AspN has a pH optimum of 8-9 and is inhibited by EDTA

4. LC-MS/MS Characterization of Yeast Extract Digest

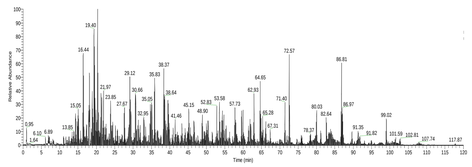


Fig 3 A. Base Peak Chromatogram; Yeast extract digested for 1 hour with recombinant AspN

	Recombinant		Native	
	1 Hr	18 Hrs	1 Hr	18 Hrs
Proteins (1% FDR)	1043	756	1020	742
Unique Peptides (0.1% FDR)	6445	3833	6338	3797

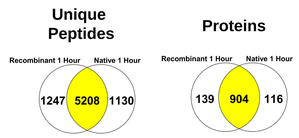


Fig 3 B. Proteins and peptides identified after digestion of yeast extract with native or recombinant AspN.

Native AspN from *Pseudomonas fragi* (2 µg; Promega V1621) and recombinant AspN from *Stenotrophomonas maltophilia* (10 µg, Promega CS1990B01) were reconstituted in 50 µL of nanopure water. 25 µg of yeast extract (Promega V7341) was reduced with DTT, alkylated with iodoacetamide and digested with 0.5 µg (1:50) of AspN for 1 or 18 hours at 37°C in a buffer containing 50 mM Tris pH 8 and a final concentration of 2M Urea. 500 ng of digested extract was analyzed by LC-MS/MS on a Q Exactive with a 2 hour gradient. Data were searched in Mascot (UniProt *S. cerevisiae* database) for cleavage at Aspartic acid and subsequently further processed with with Scaffold.

Protein and peptide identifications are similar with native and recombinant AspN

5. Sequence Specificity and Digestion Efficiency

Cleavage Specificity

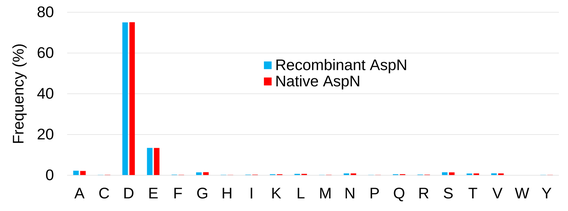


Fig. 4A. N-terminal amino acid frequency of yeast peptides identified in a "No-enzyme" search.

Cleavage Efficiency

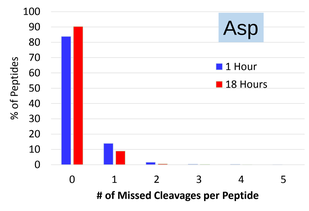


Fig. 4B. Digestion Efficiency at Aspartic Acid

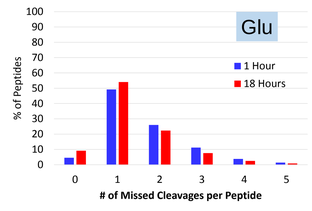


Fig. 4C. Digestion Efficiency at Glutamic Acid

Yeast extract was digested and analyzed by LC-MS/MS as in panel 4 above. Data were searched with Mascot using a "No-enzyme" approach and Scaffold output was further refined within Excel to determine the N-terminal amino acid frequency (cleavage specificity) as well as efficiency of cleavage of peptides containing Aspartic or Glutamic acid.

Recombinant AspN cleaves N-terminal to D in a highly efficient and specific manner

6. Missed Cleavage Analysis

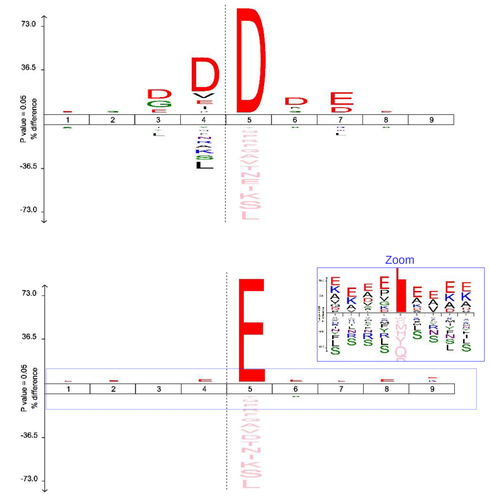


Figure 5. IceLogos for missed cleavage analysis at both D and E residues were generated using peptides identified in the yeast extract analysis described in panel 4. Sequence-aligned peptide lists for input into IceLogo were generated using in-house software in which identified peptides were "extended". The dashed line indicates the missed cleavage site.

Missed-D cleavage occurs mainly when the cleavage site is preceded by D residues
Missed-E cleavage is less predictable. Often due to nearby E, A, K and P

7. Shorter Digestion Times Result in Higher Specificity

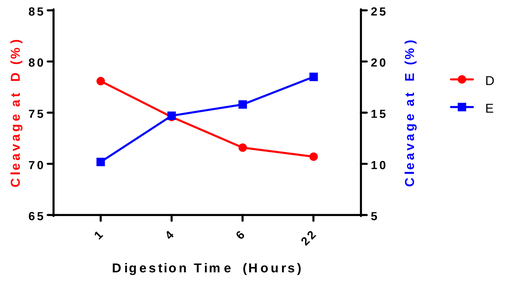


Fig 6. Time course digestion of yeast extract with rAspN: Effect on specificity.

Yeast extract (Promega V7341) was reduced with DTT, alkylated with iodoacetamide, and digested with 0.5 µg (1:50) of rAspN for various time points hours at 37°C in a buffer containing 50 mM Tris pH 8 and 2M Urea. 500 ng of digested extract was analyzed by LC-MS/MS on a Q Exactive with a 2 hour gradient. Data were searched in Mascot and were subsequently further processed with Scaffold and Excel.

Shorter digestion times result in greater specificity for D-cleavage

8. Conclusions

- Expressed and purified recombinant protease from *S. maltophilia*
- Highly active: Efficient digestion of complex mixtures in 1 hour
- Strong preference for cleavage N-terminal to aspartic acid
- Weak cleavage at glutamic acid which increases with longer digestion times
- Missed cleavages tend to be due to nearby acidic residues

CS1990B01
(10 µg)

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