Tryp-N: a thermostable, N-terminal arginine *and* lysine specific protease for ≤ 1 hr digestion, simplified peptide fragmentation and increased MS/MS sensitivity

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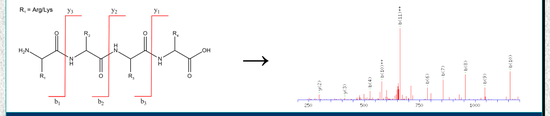
I. Overview

- A novel Arg/Lys specific N-terminal peptidase is presented.
- Spectrum simplification is effected by placing all strongly basic centers (the amino terminus, Arg and Lys) at N-termini of peptides. Most ion current is thus driven to N-terminal b-ions.
- Detection sensitivity in LOD MRM experiments is increased ~4.5x (median) relative to trypsin.
- The protease is thermophillic with a T_{opt} of ~65 °C.
- Digestion time can be very rapid, even less than 1 hr.
- The protease is highly stable and active in a wide variety of conditions including multiple buffers, pH of 5-12, detergents and salts. It withstands repeated freeze-thaw cycles.
- The protease is a metalloprotease most active in 2 mM $CaCl_2$ and 0.1 mM $ZnCl_2$.
- The addition of EDTA affords easy control of digestion (e.g. for partial digestion).

II. Introduction

Directed peptide fragmentation can generate specific ion fragment series and simplify MS/MS spectra. This is achieved by controlling where charge is located on the peptide either enzymatically or, with extra steps, by chemical modification. To produce predominantly N-terminal ions, charge must be placed at or near the N-termini of peptides. This can be most elegantly achieved using a proteolytic enzyme with N-terminal cleavage specificity at basic amino acids. This was achieved partly with Lys-N, a metalloprotease with N-terminal cleavage specificity at lysine only [1-3]. Here, we present a novel thermostable protease with equal N-terminal arginine and lysine specificity. The enzyme can be used as a direct substitute for trypsin, generates predominantly N-terminal b-ions, and completes digestions in ≤1 hr at 60 °C.



III. Materials and methods

A novel metalloprotease was expressed and purified by standard methods. Digestion efficiency was measured and conditions were optimized for temperature, incubation time, tolerance for detergents and salts, pH and preferred digestion buffers at 2% w/w enzyme. In time courses, digestion was arrested instantly by addition of EDTA. Digestion efficiency was assayed by tricine peptide gels, FITC casein assays [4] and Orbitrap XL or Velos Pro MS/MS analysis using CID or HCD fragmentation, respectively. Digestion specificity, including incorrect and missed cleavage events, was determined by Mascot and other search engines using no enzyme specificity with results filtered to 1% peptide FDR. The number of total peptides, unique peptides, cleavage residue specificity and protein IDs were calculated from these searches. For MRM LOD measurements, catalase was digested with each enzyme and a 15 point curve from 3.7 fg/injection to 1 ug/injection was quantified on a Thermo Vantage Triple Quad. Peptides were separated using a 30 min gradient and the total area for each peptide was calculated using Skyline. LOD was calculated for 17 peptides from two linear curves fit to background and the linear range of response on a log count scale.

IV. Results

1) Metal cofactor requirements

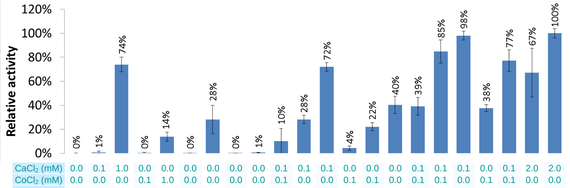


Figure 1 Proteolysis was most efficient in 2 mM $CaCl_2$ and 0.1 mM $ZnCl_2$.

2) pH optimum

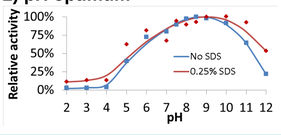


Figure 2 The metalloprotease was active in a broad pH range with an optimum around 8.5 – 10, though substantial activity remains from pH ~5 – 11. The assay was additionally run in the presence of 0.25% SDS to prevent substrate casein precipitation at low pH; few changes were observed.

3) Buffer compatibility

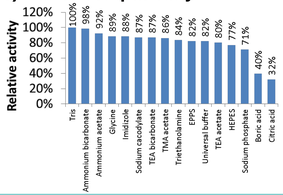
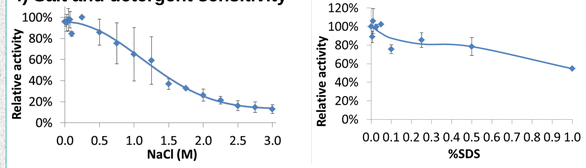


Figure 3 With the exception of buffers known to bind metals, most buffers were compatible with this enzyme. Tris, acetate and MS compatible buffers were all particularly good. To remove the effect of pH, all buffers were brought to pH 7.4 even when this was outside their normal buffering range. (TEA is triethylammonium. TMA is trimethylammonium.)

4) Salt and detergent sensitivity



Figures 4 and 5 ~80% of activity remained in 0.75 M NaCl or 0.5% SDS. Both experiments were run at 50 °C. High salt and detergent contents were dealt with using chloroform methanol extraction with subsequent determination of pellet protein content.

5) Temperature optimum

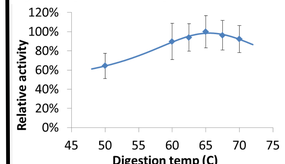


Figure 6 T_{opt} = 65 °C measured, 65.5 °C from fit curve.

6) Control of activity

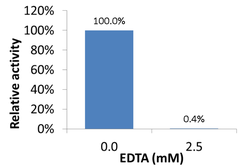
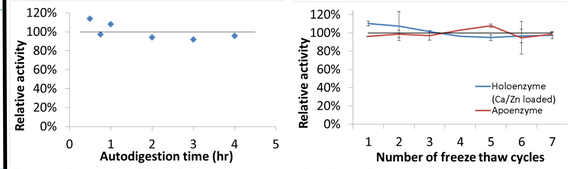


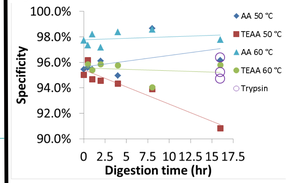
Figure 7 Addition of EDTA affords instant halt of digestion.

7) Stability & autolysis



Figures 8 and 9 > 90% of activity remained after 4 hr of autodigestion (prior to substrate addition and determination of proteolytic activity) or through 7 freeze/thaw cycles for either holo- or apoenzyme.

8) Specificity



Enzyme	Temp.	Buffer	Average specificity*
This protease	50 °C	AA	96.1%
		TEAA	94.2%
	60 °C	AA	97.9%
		TEAA	95.1%
	All	All	**95.8%**
Trypsin (ON)	37 °C	TEAB	95.4%

Figure 10 and Table 1 Cleavage specificity at K and R as a function of time and temperature, with trypsin for comparison. *Averaged over all time points. Digests with trypsin were performed overnight (16 hr). (AA is ammonium acetate. TEAA is triethylammonium acetate.)

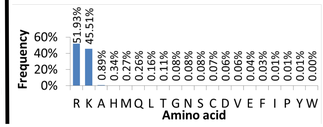


Figure 11 Pronounced specificity for arginine and lysine. 10,694 peptides from whole cell *E. coli* lysate digested at 60 °C in ammonium acetate at pH 7.4.

8) Specificity cont.

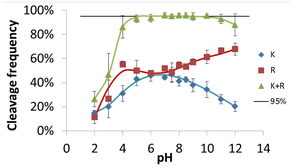


Figure 12 pH preference for K and R. Preference is equal from pH ~6-8.

9) Digestion time

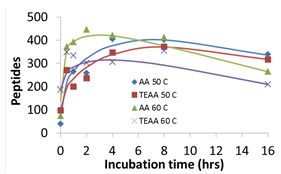


Figure 13 At 60 °C, 70-80% of detectable peptides are generated after 30 min. 0.5 – 2 hr incubation times are optimal and, although specificity is not significantly decreased, longer incubation times result in fewer detected peptides. This is likely the result of precipitation with extended exposure at elevated temperatures.

10) Comparison to trypsin

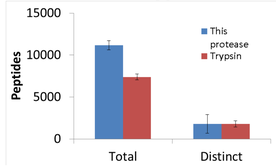


Figure 14 Whole-cell *E. coli* was digested in triplicate with trypsin and quadruplicate with this protease. ID rates were very similar.

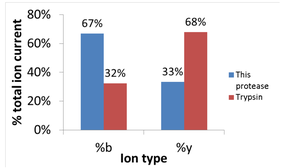


Figure 15 Digestion with this enzyme exactly inverted the preference for b- and y-ions as compared to trypsin.

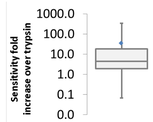


Figure 16 Decreases in MRM LOD (= increases in sensitivity) as compared to trypsin. Median fold increase in sensitivity = 4.5x. Average = 35.4x. 50% CI = 1.96x – 18.6x increase.

V. Conclusions

The enzyme exhibited a temperature optimum of 65 °C and a broad pH specificity between 5 – 12. The protease was most active in low ionic strength and MS compatible buffers, especially acetate. Enzymatic activity was most efficient in the presence of calcium and zinc. In contrast to trypsin, MS compatible or selectively cleavable detergents did not improve digestion nor number of identified peptides or proteins. Specificity for N-terminal cleavage at arginine and lysine was > ~95%, with the remaining < ~5% N-terminal mostly to larger aliphatic residues. In whole cell lysates, the numbers of identified peptides and proteins were similar to trypsin, yet generated in nearly 20x shorter digestion times. As expected, b-ion series in MS/MS spectra were generally significantly more intense than y-ions, even in multiply charged peptides (2+ and greater). Exceptions were observed in peptides containing proline or those with histidine at or near the C-terminus. In complex mixtures, a shift to lower peptide charge states was observed, likely due to close physical proximity of basic centers that reduces charging. This preference was abrogated by modification with iTRAQ, possibly due to increased available distance between charges. Efficiency of iTRAQ labeling was unchanged, as was reporter-ion intensity. This highly active metalloprotease is suitable for extremely rapid (≤1 hr) digestion of samples for immediate MS analysis. The peptides produced by this enzyme always have major basic centers at the peptide N-termini, unlike with Lys-N in which approximately half of all cleaved peptides contain internal arginine residues that can complicate fragmentation. This novel enzyme produces more easily interpretable MS/MS fragmentation spectra by generating a dominant b-ion series. Detection sensitivity is increased in comparison to trypsin due to the concentration of ion current in b ions. The speed, specificity, ease of use and overall effectiveness of this protease, combined with its ability to provide more interpretable spectra, make it a valuable component of the proteomics toolkit. **Novel Aspect:** Digest samples ~20x faster while simultaneously increasing clarity (specificity) and absolute sensitivity of MS/MS spectra.

References: [1] Nonaka, T. et al (1995) J Biochem 118, 1014-1020; [2] Taouatas, N. et al (2008) Nat methods 5, 405-407; [3] Boersema, P.J. et al (2009) Mol Cell Proteomics 8, 650-660; [4] Twining S.S. (1984) Anal Biochem. 1984 Nov 15;143(1):30-4.

Patent pending.