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Rapid and Robust Bottom-up Sample Preparation in the ProTrap XG

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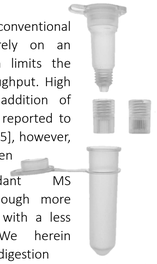
BACKGROUND

The ProTrap XG is a two-stage spin cartridge that was developed in our lab to facilitate solvent precipitation-based proteome workflows [1]. We have since developed an acetone precipitation protocol which achieves quantitative proteome recovery in minutes [2].

Following precipitation, conventional digestion procedures rely on an overnight digest, which limits the overall preparative throughput. High temperature and the addition of calcium ions have been reported to accelerate digestion [3,4,5], however, these conclusions are often based on abundant MS identifications, even though more identifications correlate with a less complete digestion. We herein present a 1-hour trypsin digestion that exploits the high activity seen at elevated temperatures in combination with the preservation effects of calcium ions, showing equivalent digestion completion to a conventional overnight digest.



SUMMARY

Bottom-up proteome analysis relies on ***robust and reproducible enzymatic digestion***, especially in the interest of accurate quantitation.

Trypsin activity assays indicate optimal enzyme activity and stability at 47 °C with 10 mM added calcium chloride.

We herein report ***enhanced proteome digestion following 1 hour of trypsin digestion at 47 °C in the presence of 10 mM calcium ions***, based on quantitative bottom-up MS analysis of fully-cleaved peptides.

Semi-tryptic cleavage and carbamyl modification are not accelerated in the rapid digest, relative to the conventional digest.

ACKNOWLEDGEMENTS

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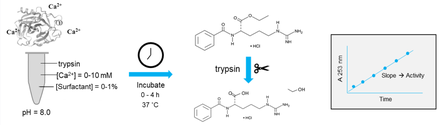
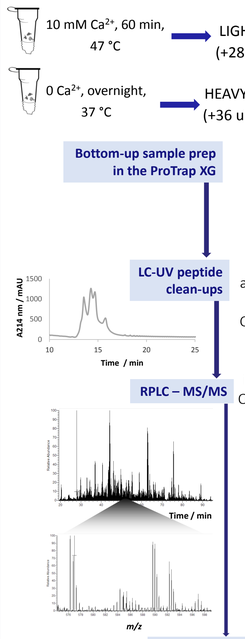
METHODS

Trypsin Activity Assays



Benzoyl arginine ethyl ester (BAEE) assays were used to determine trypsin activity. Trypsin was combined with 50 mM Tris buffer (pH 8.0), and 0 or 10 mM $CaCl_2$ at 37 °C-67 °C. Enzyme samples were either combined with BAEE reagent and assayed immediately (initial activity), or incubated for times ranging from 0.5-4 hours. At the indicated time point, an aliquot of the aging trypsin sample was combined with BAEE reagent to determine the residual activity, compared to the initial activity of a no-additive control. All slopes were normalized to initial activity with no additives.

Bottom-Up LC-MS/MS



An *S. cerevisiae* lysate was recovered by rapid precipitation, re-solubilized and digested with 50:1 trypsin in TEAB buffer (pH 8.0) under the indicated conditions in the ProTrap XG. The rapid digest was dimethyl labeled "light", the conventional digest was labeled "heavy".

Labeled digests were pooled and subject to reversed phase clean-ups with UV detection. Collected fractions were dried down, re-solubilized in 5% ACN/ 0.1% formic acid, and injected to RPLC-MS/MS on a Q Exactive Hybrid-Quadrupole Orbitrap mass spectrometer.

MS/MS spectra were searched three times in MaxQuant with full trypsin specificity, semi-trypsin specificity, and including carbamyl modifications. Bottom-up peptide identifications were filtered based on number of missed cleavages, and qualitatively and quantitatively compared across digestion conditions.

RESULTS: *Trypsin Activity & Stability*

Initial trypsin activity is optimized at 47 °C, but denaturation is accelerated at elevated temperature

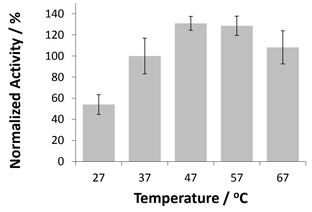


Figure 1. Trypsin activity as a function of temperature determined by BAEE assay.

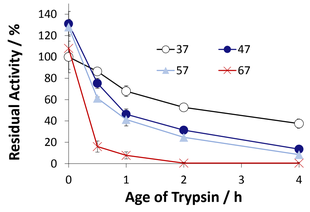


Figure 2. Trypsin activity measured by BAEE assays across 4 hr at 37-67 °C.

Calcium ions shift optimal temperature and enhance enzyme stability

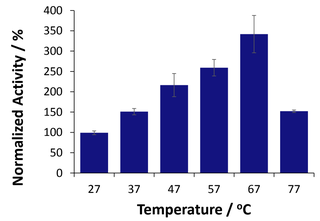


Figure 3. Trypsin activity as a function of temperature in the presence of 10 mM calcium chloride.

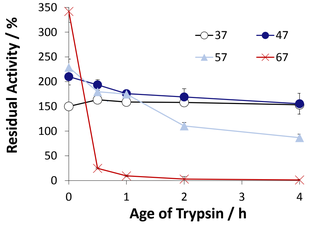
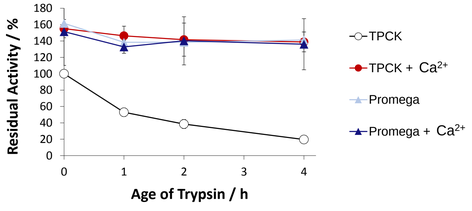


Figure 4. Trypsin activity measured across 4 hr at 37-67 °C in the presence of 10 mM calcium chloride.

TPCK-treated trypsin with calcium ions exhibits equivalent activity and stability to MS sequencing grade trypsin

Figure 5. Trypsin activity measured across 4 hr at 37 °C. TPCK-treated trypsin with 10 mM added calcium ions demonstrates equivalent activity and stability to Promega Sequencing Grade Trypsin. The same sequencing grade trypsin does not benefit further from added calcium ions.



RESULTS: *Quantitation by bottom-up LC-MS/MS*

Bottom-up peptide and protein IDs reveal high overlap between rapid and conventional digests

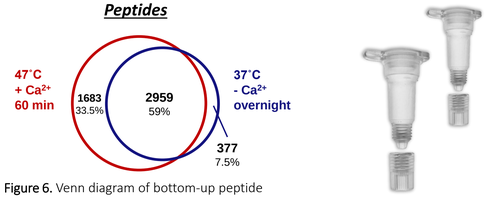


Figure 6. Venn diagram of bottom-up peptide identifications from rapid and conventional digests

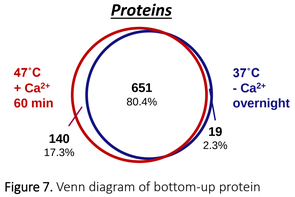


Figure 7. Venn diagram of bottom-up protein identifications from rapid and conventional digests.

Quantitative missed cleavage analysis shows fully-cleaved peptides are most abundant in rapid digest

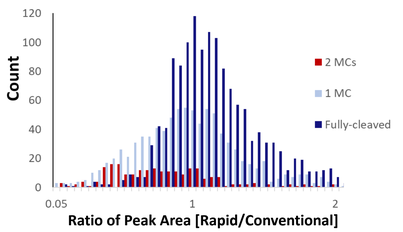


Figure 8. Histogram of peptides' relative abundance in rapid digested sample vs. conventionally digested sample. Fully-cleaved peptides have greater abundance in the rapid digest, while miss-cleaved peptides have greater abundance in the conventional digest.

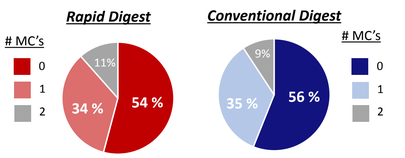


Figure 9. Missed cleavage analysis of peptides identified by bottom-up LC-MS/MS following rapid digestion (1 hr at 47 °C with 10 mM calcium chloride) and conventional digestion (overnight at 37 °C with no added calcium chloride).

Semi-tryptic cleavage and carbamyl modification rates are not accelerated in the rapid/ high-temperature digest

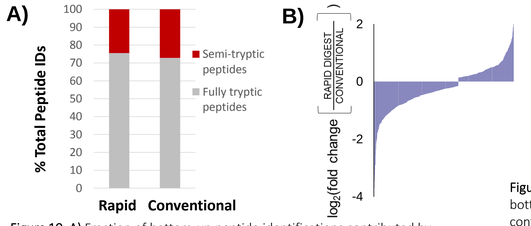


Figure 10. A) Fraction of bottom-up peptide identifications contributed by semi-tryptic peptides. **B)** Relative quantitation of semi-tryptic peptides in the rapid digest vs. the conventional digest.

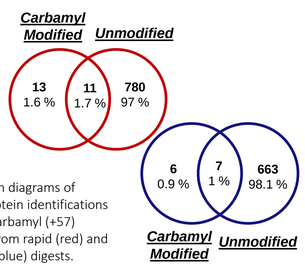


Figure 11. Venn diagrams of bottom-up protein identifications containing a carbamyl (+57) modification from rapid (red) and conventional (blue) digests.

DISCUSSION & CONCLUSIONS

- Initial trypsin activity is optimized at 47 °C, but the addition of calcium ions shifts the optimal temperature to 67 °C.
- The addition of ***calcium ions enhances enzyme stability*** across a digestion period, affording the use of elevated temperatures for proteome digestion.
- Bottom-up LC-MS/MS shows ***greater peptide and protein identification rates*** from the rapid (1 hour, 47 °C, +10 mM Ca^{2+}) digest compared to the conventional overnight digest.
- Relative quantitation revealed a ***greater abundance of fully-cleaved peptides in the rapid digest*** compared to the conventional digest, suggesting ***enhanced digestion efficiency in just 1 hour***. Miss-cleaved peptides are frequently identified with greater abundance in the conventional overnight digest.
- Relative quantitation shows most semi-tryptic peptides have a greater abundance in the conventional overnight digest.
- Urea-driven carbamyl modification was determined to occur at a negligible rate; however, longer digests at the elevated temperature, or in different buffers could result in greater modification rates.

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