On the Advantages of Admixed Lys-C/Lys-N Digests for Proteome Depth and DeNovo Sequencing

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Overview
The preparative cornerstone of bottom-up or shotgun proteomics is enzymatic protein digestion to peptides. Digestion has many advantages over intact approaches, including increased fragmentation efficiency and availability of robust database search methods vs. in-silico peptide sequences to assign fragment ion spectra. We examine the utility of combining LysC and LysN proteolytic digests, which doubles digest complexity but increases the probability of peptide identification for isotopic and isomeric peptides.

Introduction
Most enzymatic proteases specifically target a particular amino acid residue at a particular terminus.

Heck1 pioneered the use of LysN, with advantageous b- and c- fragment ion series via HCD and ETD. We rationalized that combining LysC and LysN digests (LysCN) would provide isotopic and isomeric peptide pairs, potentially providing more complete by and/or c/z fragment ions for superior scoring, terminal characterization and de novo sequencing.

Method
Sample preparation
A 10 protein mix, yeast lysate, and human K562 lysate were each solubilized in 8M urea, 50mM ammonium bicarbonate, then reduced and alkylated using DTT (5mM) and propionamide (10mM), respectively. Samples were adjusted to <1mM urea with 50mM ammonium bicarbonate pH 8.0 and digested overnight at 37°C using 1:25-50 protease to protein ratios of LysC and LysN in separate reactions. The resulting digests were quenched and cleaned up on stage tips. Terminal enrichment was done using a positional proteomics’ approach as previously reported.

Mass Spectrometry and Liquid Chromatography
NanoLC-MS/MS analysis was run on an Acquity M-Class using an in-house packed C18 reversed-phase column, with either a LTQ Orbitrap Velos or Fusion mass spectrometer using both High-High (HCD) and High-Low (CID) methodologies.

Database Searching and Statistical Evaluation
.$RAW.$ files were searched using Byonic to a 1% FDR and post-processed using Combyne, Matlab, and Excel. The processed data was visualized with the Byonic viewer and Excel.

Results
Retention Time Shift
Differences in pI for isotopic peptides (Scheme 1) resulted in significant retention time shifts. Universally, the peptides containing C-terminal K residues eluted earlier than the N-terminal K isomers. While some instances of co-elution were observed, they were not amenable to co-fragmentation.

Proteome Depth
With complex mixtures, e.g. yeast and K562 lysates, the doubled complexity of LysCN admixed digests overwhelms the instrument duty cycle. This saturation results in minimal differences in proteome depth vs. independent digests at typical chromatographic timescales, but significant improvements in protein assignment scores. The result metrics for 90 min, and 120 min. chromatographic runs of LysC, LysN, and the combined LysCN in Table 1 below demonstrate the importance of understanding sample complexity when selecting analytical conditions for admixed digests.

Future
• Identify chromatographic conditions that enable co-isolation of LysC and LysN peptide pairs
• Apply strategy to areas which would benefit most:
  • De novo sequencing of antibodies
  • Characterization of lysine post-translational modifications
  • Identification of peptide N-termini

Conclusions
When LysC and LysN digests are combined, the LysC and LysN peptide pairs typically elute several minutes apart under reversed phase chromatographic conditions. This lack of co-elution doubles the sample complexity when LysC and LysN digests are combined, and highlights the importance of adjusting the chromatographic separation to suit the complexity of the sample.

The average log probabilities of identified proteins increased, and low scoring peptides can be rescued, when both LysC and LysN isomeric peptides are present and provide complementary information.

References

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