Highly Specific and Autoproteolytically Resistant Trypsin for Accurate Protein Mass Spec Analysis

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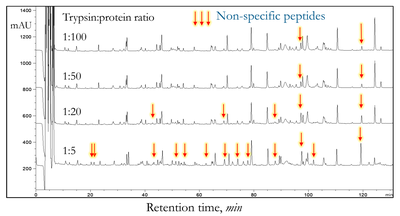
1. Introduction

Trypsin is the most popular protease in protein mass spec field owing to robust performance, optimal distribution of tryptic cleavage sites in proteins and strong charge of trypsin generated peptides. To meet requirements of protein mass spec analysis, trypsin must demonstrate high cleavage specificity and stability. In this study, we investigated trypsin properties in detail. The commonly used commercially available Proteomics and MS grade trypsins showed low, but detectable non-specific cleavage specificity. They also demonstrated prominent autoproteolysis. Non-specific and tryptic autoproteolytic peptides became abundant if large trypsin quantities were used. This compromised protein mass spec analysis. To address the problem, we developed new trypsin that was free of non-specific activity, had high autoproteolytic resistance and increased proteolytic efficiency.

2. Non-specific cleavage activity in MS grade trypsins

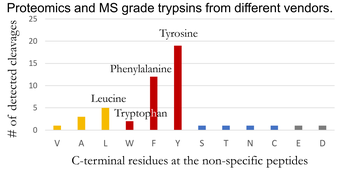
Non-specific cleavage activity in MS grade trypsin

Panitumumab antibody was digested overnight at indicated trypsin:Ab ratio and analyzed with RP-HPLC-UV (Agilent 1200).



Non-specific cleavages identified in the tryptic digest

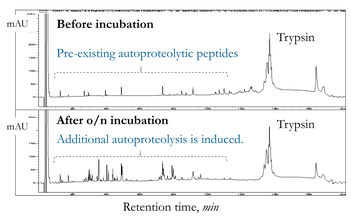
The non-specific peptide peaks detected in RP-HPLC-UV were assigned with LC-MS. This graph shows the frequency at which different residues were found at the non-specific peptide C-termini. The same non-specific cleavages were found in Proteomics and MS grade trypsins from different vendors.



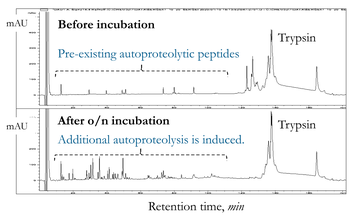
Proteomics and MS grade trypsins contain non-specific, chymotrypsin-like activity. Non-specific cleavages become abundant at large trypsin quantities typically used in peptide mapping (i.e., 1:5-1:10 trypsin:protein ratio).

3. Autoproteolysis of Proteomics and MS grade trypsins

Autoproteolysis of Proteomics grade trypsin
RP-HPLC-UV analysis



Autoproteolysis of MS grade trypsin
RP-HPLC-UV analysis



Proteomics and MS grade trypsins show prominent level of pre-existing and digestion-induced autoproteolytic tryptic peptides. This indicate the need for improvement of chemical modification method used to suppress trypsin autoproteolysis.

4. Development of new, improved trypsin

Research stage

Screening of trypsins from different organisms to identify the most active and stable variant

Production

Multiple purification and modification steps

Trypsin expression in a microbial host → Dispensing and lyophilization

All production steps are closely controlled to assure high purity, cleavage specificity, activity and lot-to-lot reproducibility of trypsin.

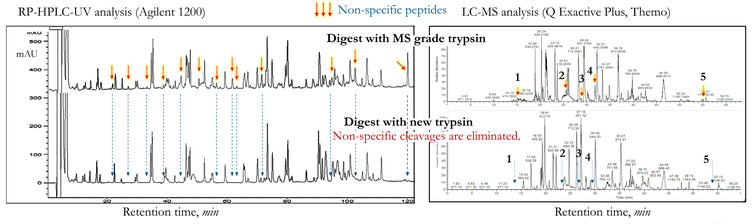
After manufacturing, new trypsin is subjected to rigorous QC analysis to assure that the critical product requirements are met.

- The most active and stable trypsin variant is used to develop new trypsin. New trypsin is produced in a highly pure, active and stable recombinant form.
- New trypsin is free of contaminating proteases.
- Novel trypsin modification method was developed to significantly increase trypsin autoproteolytic resistance.
- New trypsin is subjected to multiple purification steps to ensure superior purity.

New trypsin preparation procedure is optimized to ensure full elimination of non-specific cleavage activity, maximal suppression of autoproteolysis, enhanced proteolytic efficiency and high lot-to-lot reproducibility.

5. New trypsin is free of non-specific cleavage activity.

Test for cleavage specificity: New trypsin vs MS grade trypsin
Panitumumab antibody was digested overnight with trypsins at 10:1 ratio.



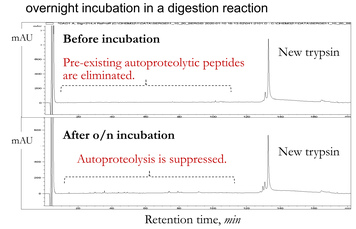
Non-specific (semi-tryptic) peptides, #

1- (F)CQHFDHPLAFGGGTK
2- (F)TFTISSLQPEDIATYFcQHFDHLPLAFGGGTK
3- (F)IFPPSDEQLK
4- (Y)FCQHFDHLPLAFGGGTK
5- (F)SGSVMHEALHNHYTQK

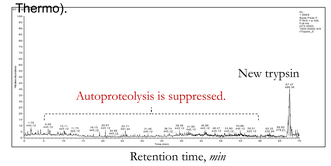
New trypsin is free of non-specific cleavage activity.

6. New trypsin is autoproteolytically resistant.

Test for autoproteolysis
RP-HPLC-UV analysis of new trypsin before and after overnight incubation in a digestion reaction



LC-MS analysis of new trypsin (Q Exactive Plus, Thermo).

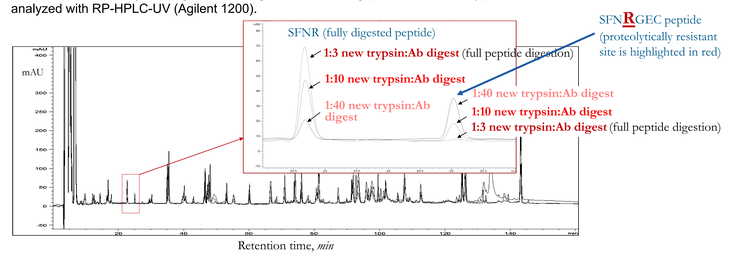


Our preparation procedure and the improved trypsin chemical modification method ensure elimination of pre-existing and digestion-induced autoproteolytic tryptic peptides.

7. Efficient digestion of proteolytically resistant sites

Digestion of a proteolytically resistant site with increasing quantities of new trypsin

Panitumumab antibody was digested overnight with increasing quantities of new trypsin and analyzed with RP-HPLC-UV (Agilent 1200).

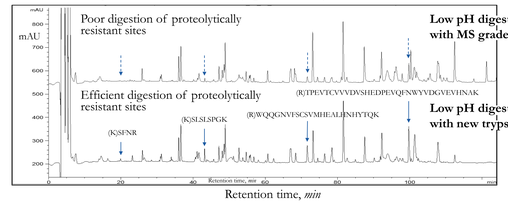


Complete digestion of proteolytically resistant sites can be achieved using large quantities of new trypsin. Proteomics and MS grade trypsins cannot be used in this method because they induce excessive protein degradation at the extra-large trypsin quantities used in the method.

8. Efficient digestion with new trypsin at acidic pH

Enhanced digestion at acidic pH with new trypsin

Panitumumab antibody was digested at mildly acidic conditions using 1:5:5 Lys-C:trypsin:Ab ratio and analyzed with RP-HPLC-UV (Agilent 12300).



AccuMAP™ Low pH Digestion kit suppresses artificial PTMs commonly induced during protein mass spec sample preparation including:

- Deamidation (succinimide intermediate is stabilized with the kit).
- Asp isomerization
- Disulfide bond scrambling
- Methionine oxidation

We have recently developed AccuMAP™ kit for protein digestion at low pH. The kit suppresses artificial non-enzymatic PTMs. By supplementing the kit with new trypsin, we significantly improved the kit digestion efficiency at acidic conditions.

AccuMAP™ kit in combination with new trypsin provides the conditions for efficient and accurate characterization of non-enzymatic modifications in biotherapeutic proteins.

9. Conclusions

We have developed new trypsin that addresses shortcomings of existing Proteomics and MS grade trypsins.

- It is free of non-specific cleavage activity commonly found in Proteomics and MS grade trypsins.
- Using new chemical modification method, we significantly improved new trypsin autoproteolytic resistance comparing to the existing Proteomics and MS grade trypsins.
- Proteolytic efficiency of new trypsin was increased. New trypsin can be used to effectively digest proteolytically resistant sites.
- Owing to extensive purification, new trypsin shows superior purity. It is also free of contaminating proteins of animal origin.

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