Enhancing trypsin digestion with Lys-C and Arg-C proteases

Sergei V. Saveliev[1], Laurie Engel[1], Mike Rosenblatt[1], Richard Jones[2], Michael Ford[2], Ravi Amunugama[2], Dave Allen[2] and Marjeta Urh[1]

[1]Promega Corporation, Madison, WI; [2]MS BioWorks, LLC, Ann Arbor, MN *email: sergei.saveliev@promega.com*

Promega

1. Introduction

Owing to efficient proteolysis and particular advantages of trypsin-generated peptides, trypsin is the most widely used protease in mass spectrometry. However, it is often overlooked that trypsin has shortcomings such as incomplete proteolysis, poor digestion of tightly folded proteins or an inability to cleave arginine and lysine residues adjacent to proline. These shortcomings negatively affect mass spec analysis, particularly advanced applications such as protein quantitation.

We show here that Lys-C and Arg-C proteases address trypsin shortcomings. By supplementing trypsin with Lys-C we have achieved ≥95% digestion efficiency as judged by the level of completely digested peptides. In addition, by utilizing the remarkable resistance of Lys-C to denaturing conditions we have adapted this trypsin/Lys-C mix to digest a proteolytically resistant protein. Finally, we demonstrate the unique utility of Arg-C protease based on its ability to cleave the residues adjacent to proline.

2. Recombinant Lys-C protease

rLys-C cleavage site

NNNNNNK NNNNNNNNNNNN

Expression in E.coli

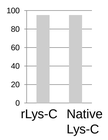
Purification

Dispensing and lyophilization

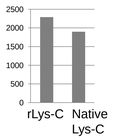
rLys-C manufacturing

Benchmarking against native Lys-C

Specific cleavages (%) in yeast protein extract digests

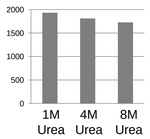


Peptide identifications in yeast protein extract digests



Tolerance to denaturing conditions

Peptide identifications in human protein extract digested with rLys-C at increasing Urea concentrations



The recombinant protease is as active as native Lys-C and tolerates strong denaturing conditions

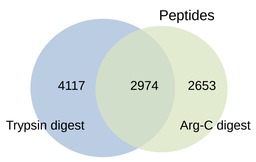
rLys-C is derived from Pseudomonas aeruginosa and expressed in E. coli

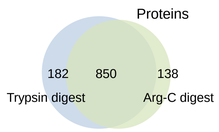
3. Clostripain (Arg-C) protease

Arg-C cleavage sites

NNNNNR NNNNNK NNNNNNNN

Yeast proteomic analysis using parallel digestion with trypsin and Arg-C





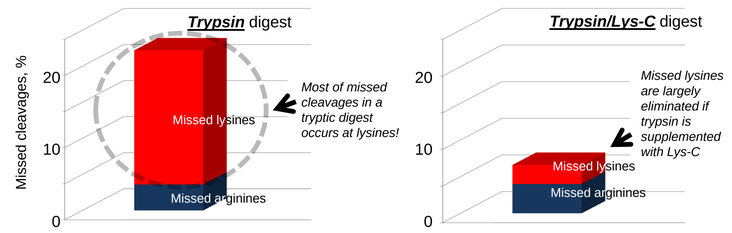
Parallel digestion with Arg-C can be used to increase protein or proteome coverage

Clostripain (Arg-C) protease is purified from Clostridium histolitycum. The protease preferentially cleaves at C-terminal side of arginine (R) residues. It also cleaves at lysine (K) residues albeit less efficiently.

4. Advantage of Lys-C-supplemented trypsin

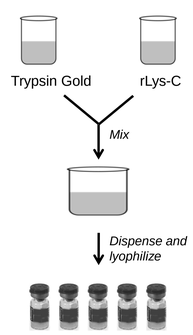
Typically, ~20-30% peptides in a tryptic digest are incompletely digested. Incomplete digestion is a major source of irreproducibility of mass spec analysis or inaccurate protein quantitation. In contrast to a logical assumption, missed R and K in a tryptic digest are not represented in equal amounts. Most of missed cleavages occur at lysines.

Missed cleavages (incompletely digested peptides) in yeast total protein digests

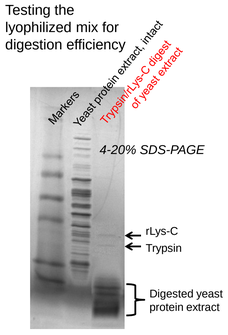


Supplementing trypsin with Lys-C dramatically improves digestion efficiency

5. Preparation of trypsin/rLys-C mix



Testing the lyophilized mix for digestion efficiency

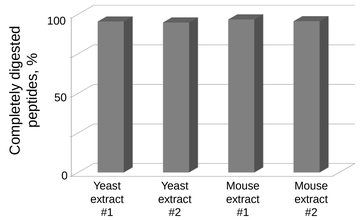


The mix efficiently digests a cell protein extract

Note that trypsin and rLys-C do not digest each other because both proteases are proteolytically resistant

6. Validation of Trypsin/rLys-C mix

Digestion of yeast and mouse protein extracts with Trypsin/rLys-C mix

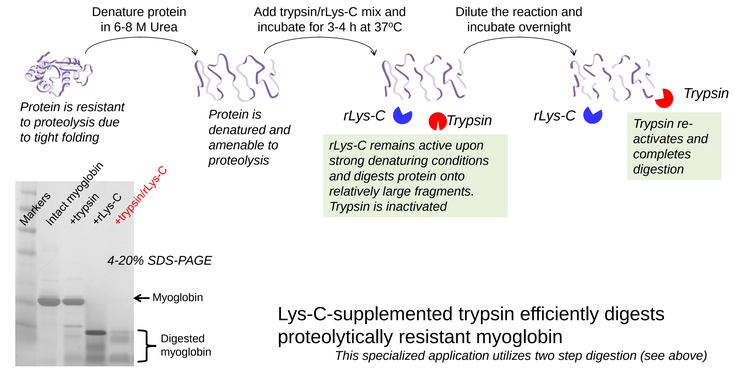


Trypsin/rLys-C mix consistently provides ≥95% digestion efficiency (expressed as % of completely digested peptides)

The remaining missed cleavages represent a few percent of total peptide population and predominantly occur at (R/K)/(D/E) sites or at peptide N-termini

Experimental conditions: total yeast and mouse protein extracts were digested with trypsin/rLys-C mix at 37ºC overnight . The digestion was performed under conventional trypsin-amenable (non- or mildly denaturing) conditions. The digests were analyzed with LTQ Orbitrap Velos

7. Additional, specialized application of Trypsin/rLys-C mix: digestion of tightly folded, proteolytically resistant proteins



Lys-C-supplemented trypsin efficiently digests proteolytically resistant myoglobin

This specialized application utilizes two step digestion (see above)

8. Arg-C advantage

Cleavage of RP and KP sites with Arg-C in yeast total protein extract

Protease	Unique peptides	Cleavages at arginines	Cleavages at lysines	**Cleaved RP sites**	**Cleaved KP sites**
Trypsin	4481	37%*	61%*	**0%**	**0%**
Arg-C	3374	56%	41%	**97%**	**44%**

**Difference in the share of arginine and lysine cleavages in trypsin digest reflects natural unequal distribution of these residues in proteins rather than trypsin cleavage preference toward lysines*

Unlike trypsin, Arg-C efficiently cleaves at arginine and, to a lesser extent, lysine residues adjacent to proline

9. Conclusion

- ❑ **Supplementing trypsin with rLys-C improves digestion efficiency**
  ≥95% peptides are completely digested
- ❑ **Trypsin/Lys-C mix can also be used to digest tightly folded, proteolytically resistant proteins**
  This specialized application utilizes Lys-C tolerance to protein denaturing conditions
- ❑ **Arg-C protease compliments trypsin by cleaving arginine and lysine sites before proline**
  Arg-C can also be used to improve proteome coverage