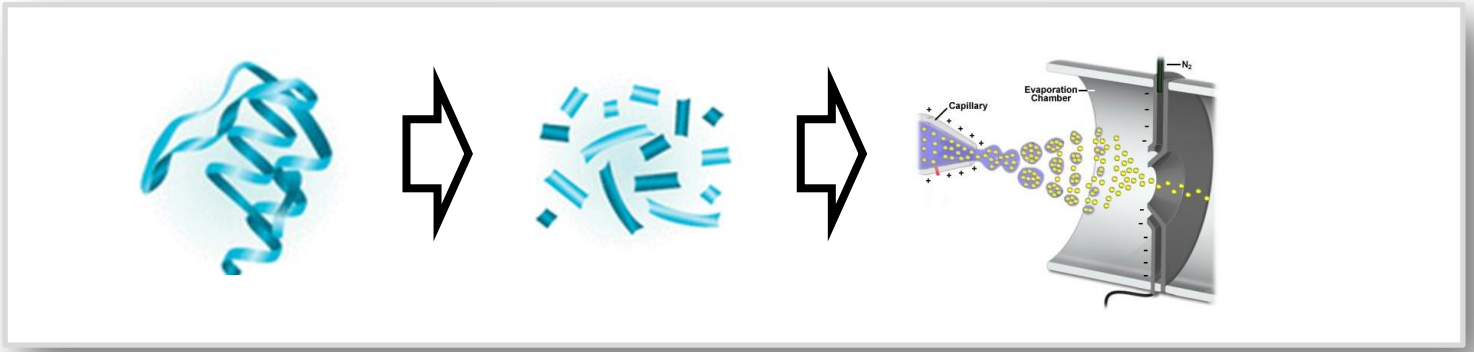
Enhanced Protein Mass Spectrometry Analysis with Trypsin/Lys-C Mix

Sergei Saveliev, Ph.D.
Sr. Research Scientist II, Promega Corporation

Trypsin/Lys-C Mix for Protein Mass Spec Analysis



Trypsin/Lys-C mix brings trypsin performance to the next level:

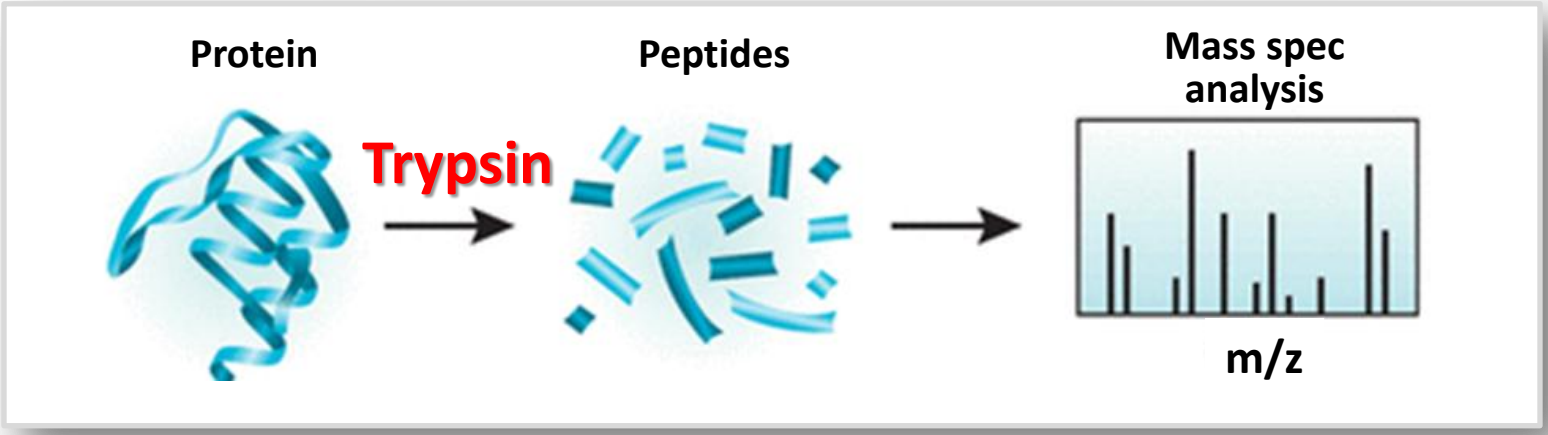
- *Minimizes undigested cleavage sites, which commonly occur in trypsin digests.*
- *Efficiently digests tightly folded proteins, which are resistant to trypsin.*

The result is more efficient and reproducible mass spec analysis.

Outline

- Key role of trypsin in protein mass spec sample preparation
- Trypsin/Lys-C advantage
  - Effect of supplementing trypsin with Lys-C
  - Application data from outside laboratories
- Recommendations for use

Role of Trypsin in Protein Mass Spec
Digestion of Proteins into Analyzable Peptides



- **Trypsin is the most popular protease used in mass spec field.**
- **<u>Majority of protein mass spec analyses use trypsin.</u>**

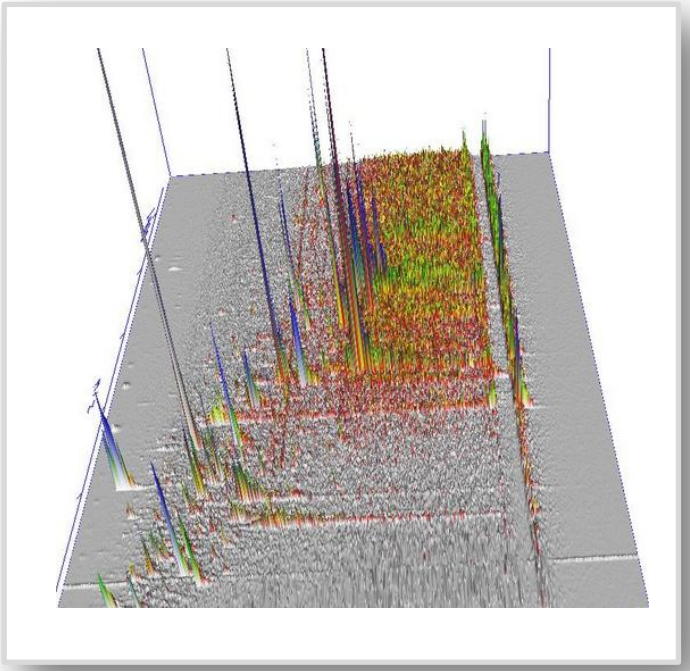
Trypsin advantages:

- *High proteolytic activity*
- *Exceptional cleavage specificity*
- *Tryptic peptides have optimal size and charge*

Growing Demand for More Efficient MS Analysis
Proteolysis is a Becoming Major Obstacle

The requirements of more efficient protein mass spec analysis include:

- Higher protein and proteome coverage
- Higher reproducibility of the analysis
- Accurate protein quantitation



Proteolysis is becoming a major obstacle toward further improvement of protein mass spec analysis. Why?

Trypsin Still Has Shortcomings

- ✓ Digestion with trypsin is rarely complete.
- ✓ Tightly folded proteins are resistant to trypsin.
- ✓ Many popular sample preparation reagents inhibit trypsin.

Compromised analytical reproducibility and quantitation

Missed Cleavages in Trypsin Digest

Trypsin Does Not Cleave As Efficiently at Lysine Residues

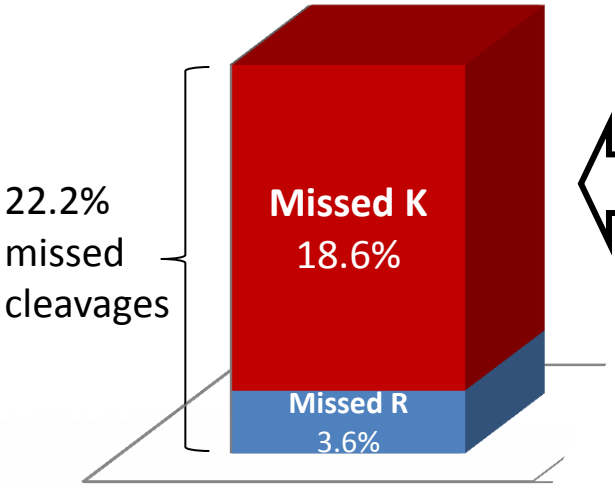
Promega

8028	SPEHLIQYLFAELGTSGSVDGQK
8031	SVSADAEA[K]PTDDIAEALGELSLK
8032	TGFQATVGK
8033	TIFSNIQDIAEK
8034	TNNPELAGDR
8035	YILEYVTCK
8036	DIFTTLVSPE[L]STCK
8037	EATEA[R]ETEK
8039	K[Y]DVEPEDDEEDTQTEGK
8040	LLCDEYEADPFIR
8041	TGNTALHWASLNGK
8042	AELINNLGTIAK
8043	AILFIPK
8044	APFDLFESK
8045	DDQLEYLEEK
8046	DFELEETDEEK
8047	DFELEETDE[K]AER
8048	DSSMSSYMSSK
8049	EEVQEIEELNK
8050	EILGDQVEK
8051	ELISNASDALDK
8052	EVPIPEEEKKDEEKKDEEK
8053	GVVDSEDLPLNLSR
8054	HFSVEGQLEFR
8055	HSEFVAYPIQLVVTK
8056	LEEVDEEEE[K]P[K]
8057	LF[K]DDQLEYLEEK
8058	LGVHEDTQNR

Missed cleavages

Peptides identified in yeast protein overnight digest

Typically, trypsin leaves behind 10-30% missed (undigested) sites.



Unexpectedly, the majority of missed cleavages occurs at lysine sites.

Composition of Missed Cleavages in Trypsin Digest

Higher Percentage of Missed Lysines vs. Arginines

Promega

Protein Extract and Preparation Method	**Total Missed Cleavages**	**Missed K**	**Missed R**	
Yeast extract #1 (Urea/protease inhibitor extraction)	22.2%	18.6%	3.6%	
Yeast extract #2 (Urea extraction)	10.8%	9%	1.8%	
Mouse extract (ProteaseMAX extraction)	7.7%	6.6%	1.1%	
K562 extract (Urea extraction)	10.4%	8.8%	1.6%	
E. coli extract *(GuCl extraction)*	43.2%	37%	6.2%	

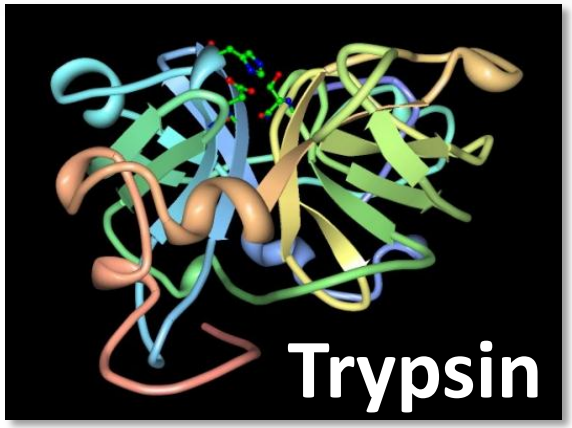
Missed K : Missed R ratio in trypsin digests = 5:1 - 6:1.

Trypsin Cleavage Specificity

Trypsin cleaves lysine sites less effectively than arginine sites

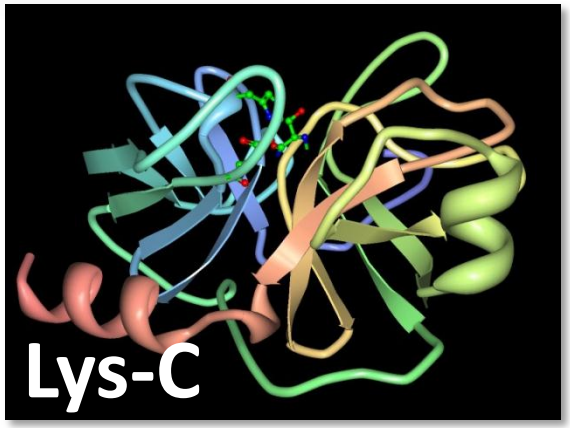
- *The lysine cleavage inefficiency is the major source of incomplete digestion*

Improving Trypsin by Supplementing with Lys-C



NNNN(K/R) NNNN

- ✓ Robust
- ✓ Very high cleavage specificity



NNNNK NNNN

- ✓ Extremely robust
- ✓ Very high cleavage specificity
- ✓ Tolerant to denaturing conditions

Lys-C addresses trypsin proteolytic inefficiency at lysine sites

Supplementing trypsin with Lys-C enhances trypsin performance without affecting the structure of the digestion products. The generated peptides are still tryptic peptides!

Trypsin/Lys-C Mix Components: Trypsin Gold

Raw trypsin

TPCK treatment

Chemical modification (methylation)

Purification

The source - porcine trypsin. Porcine trypsin is more active than bovine trypsin used by other companies.

TPCK inhibits chymotrypsin, which contaminates trypsin and compromises trypsin cleavage specificity

- *Prevents generation of pseudotrypsin, which has chymotrypsin-like activity*
- *Prevents autoproteolysis*
- *Increases thermostability (methylated trypsin remains activity at 55°C)*

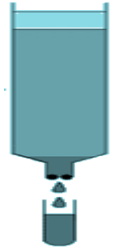
Pooling the fractions with the highest activity

Trypsin Gold

Trypsin/Lys-C Mix Components: rLys-C Protease

Pseudomonas aeruginosa Lys-C is expressed in *E. coli* and extensively purified.

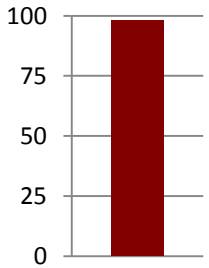
The final product is a fully functional, highly active Lys-C protease.



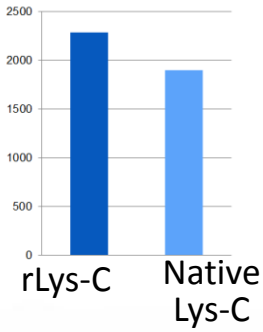
SDS-PAGE

Purified rLys-C

Specific Cleavages, %
Yeast protein digest with rLys-C

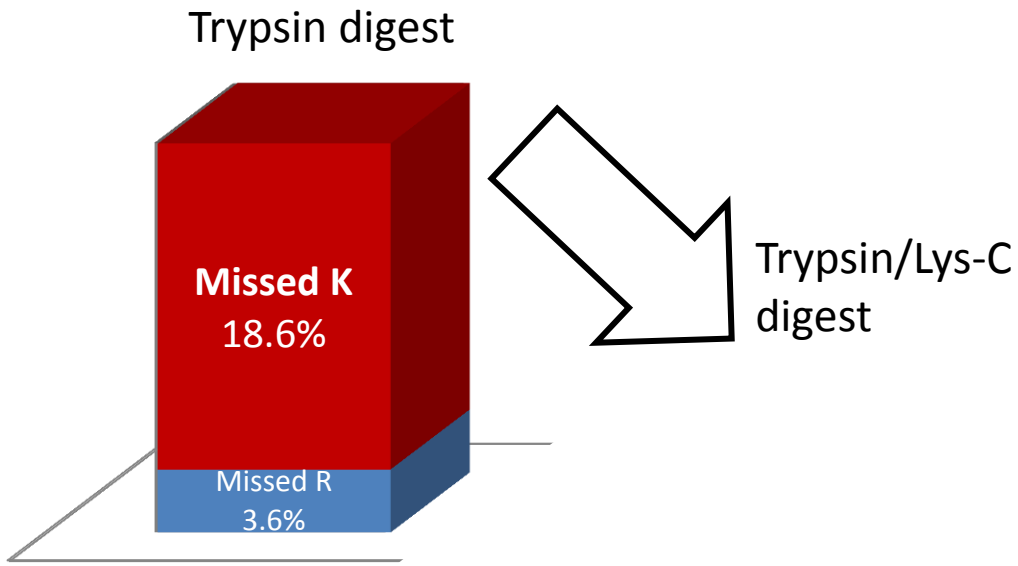


Identified Peptides
Yeast protein extract digests



Trypsin/Lys-C Mix Improves Proteolysis

Missed cleavages in yeast protein digest



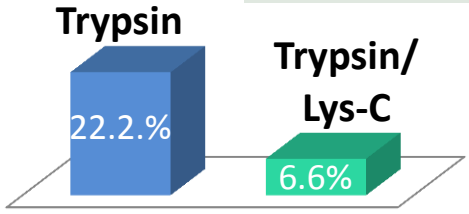
Trypsin/Lys-C digests the majority of missed lysine sites and increases overall digestion efficiency.

Improved Proteolytics Efficiency of Trypsin/Lys-C vs. Trypsin Alone in Side-by-Side Comparisons

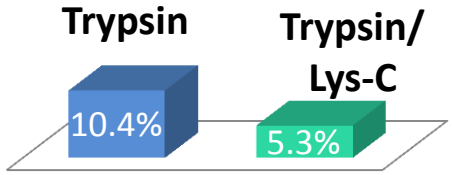
Missed cleavages *(in % of total cleavages)* ***in different samples.***

The samples were digested with Trypsin and Trypsin/Lys-C under identical conditions (overnight incubation at 37°C).

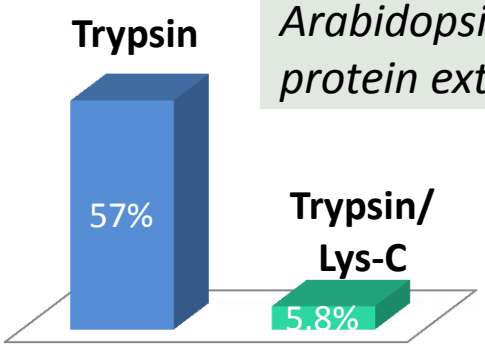
Yeast protein extract



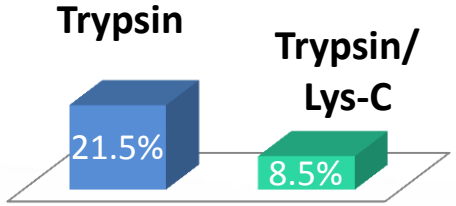
K562 protein extract



Arabidopsis protein extract



FFPE tissue protein extract



Composition of Remaining Missed Cleavages in Trypsin/Lys-C Digest

Sample	Missed K	Missed R
Yeast extract #1	2.6%	4%
Yeast extract #2	3.1%	2.1%
Mouse extract #1	2.9%	1.5%
Mouse extract #2	2.2%	1.1%

Level of missed K cleavage sites drops to the level of missed R sites.

Remaining missed cleavages

(K/R)NNNNNNNN — *N-terminal K and R*

NNNNN(K/R)(D/E)NNNNNNNNNN — *(K/R)(D/E) sites*

NNNNNNNNNNN(K/R)NNNNNNNNN — *Modified residues*

The above sites are particularly proteolytically resistant.

Trypsin/Lys-C Cleavage Specificity

Trypsin cleavage specificity

Trypsin/Lys-C cleavage specificity

Trypsin/Lys-C cleaves K and R sites with similar efficiency

- ***Lys-C compensates for trypsin proteolytic deficiency at lysine sites.***

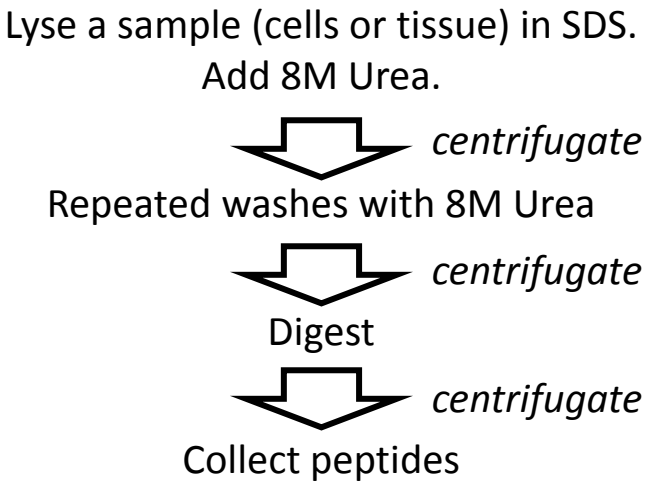
Study #1: Analysis of FFPE Skin Tissue Extract

Sample prep is difficult due to extensive protein crosslinking in FFPE tissue.

The extract was digested with Trypsin Gold or Trypsin/Lys-C using FASP protocol.

Filter-aided sample preparation method (FASP)

Wisniewski et al. Nature Methods (2009) 6:359-362



All steps are performed in a ultrafiltration device.

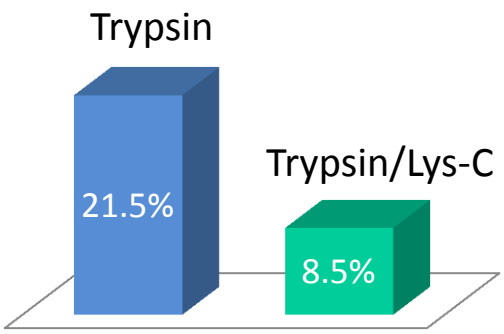
Courtesy by C. Adams, Stanford U

Study #1: Analysis of FFPE Skin Tissue Extract

Sample prep is difficult due to extensive protein crosslinking in FFPE tissue.

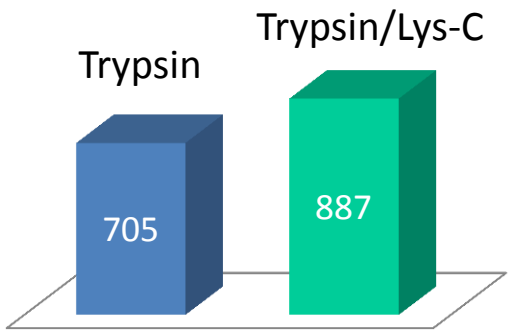
The extract was digested with Trypsin Gold or Trypsin/Lys-C using FASP protocol.

Missed Cleavages, %



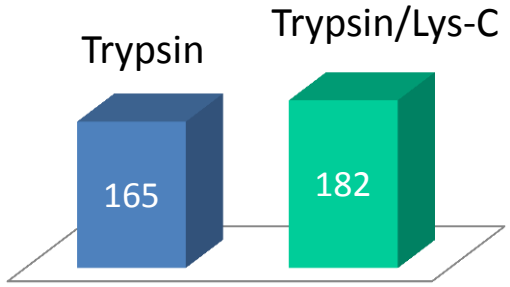
2.5 fold drop

Identified Peptides



24% increase

Identified Proteins



10% increase

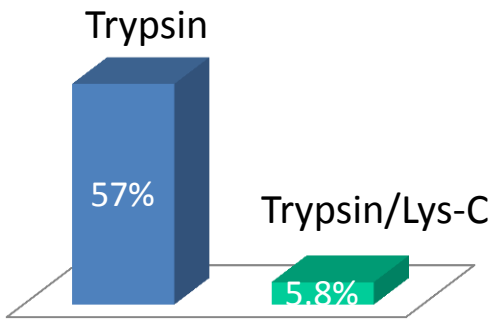
Courtesy by C. Adams, Stanford U

Trypsin/Lys-C increased number of identified peptides and proteins for 24% and 10%, respectively.

Study #2: Analysis of Arabidopsis Protein Extract

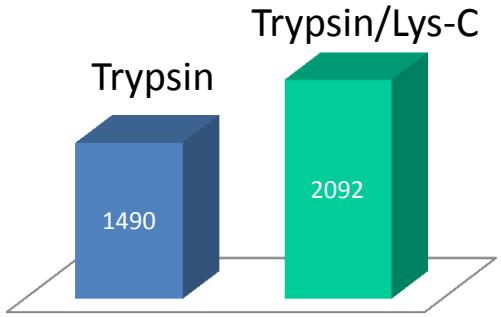
The extract was digested with Trypsin Gold or Trypsin/Lys-C overnight at 37°C.

Missed Cleavages, %



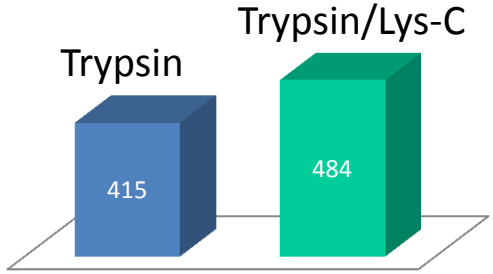
Ten fold drop in missed cleavages

Identified Peptides



40% increase

Identified Proteins



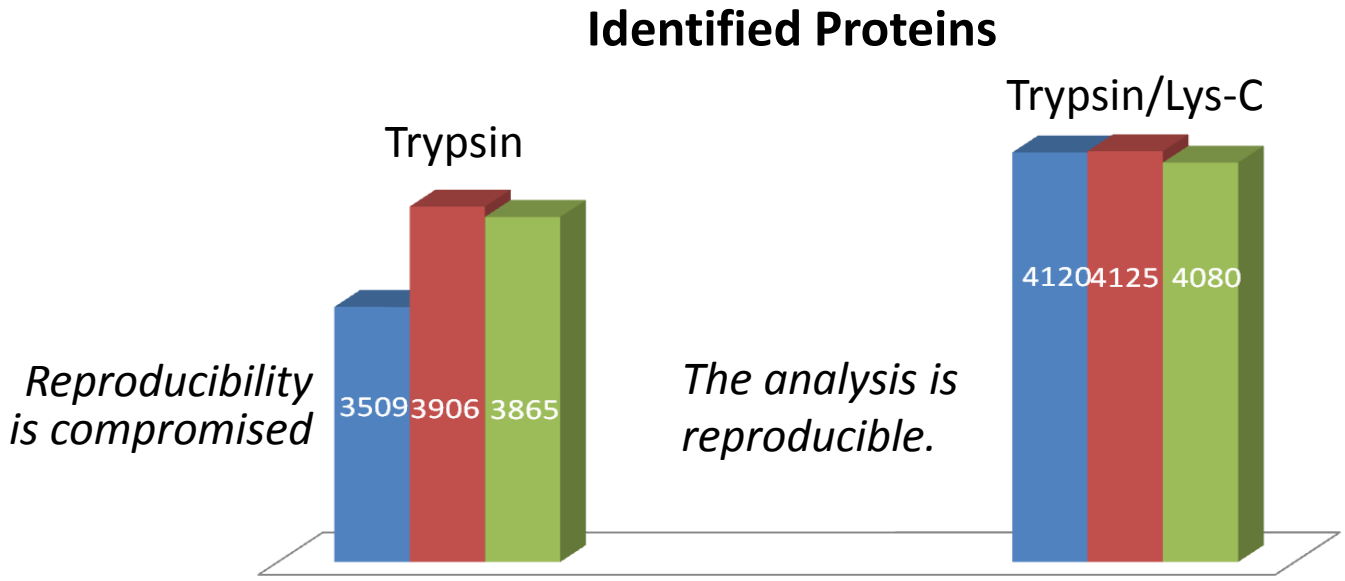
17% increase

Courtesy by B. Minkoff, U of Wisconsin-Madison

Trypsin/Lys-C increased number of identified peptides and proteins for 40% and 17%, respectively.

Study #3: Improved Reproducibility of HeLa Protein Extract Digestions

The extracts were digested with Trypsin Gold or Trypsin/Lys-C overnight at 37°C.

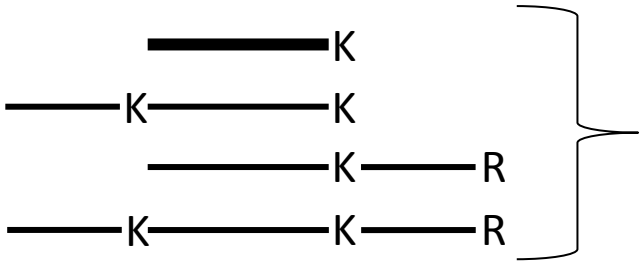


Courtesy by H. Budamgunta, Karolinska Inst

Trypsin/Lys-C improved consistency of the analysis. It also increased number of identified peptides and proteins for 20% and 10%, respectively.

Improved Protein Quantitation with Trypsin/Lys-C

Trypsin digest



Peptide of interest is incompletely digested

Protein quantitation is inaccurate due to incomplete digestion

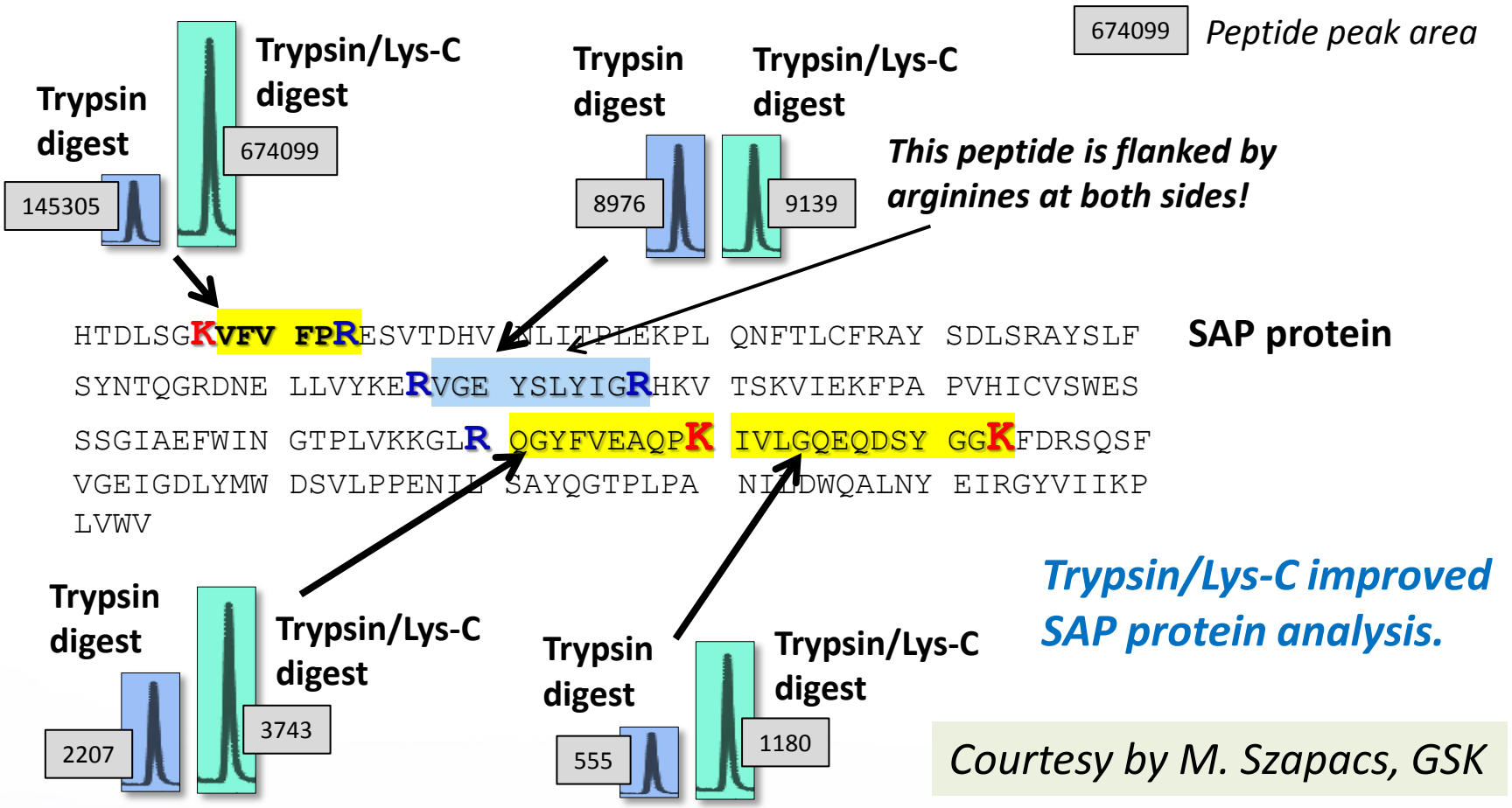
Trypsin/Lys-C Digest

Peptide of interest is completely digested

Protein quantitation is accurate

Study #4: Improved Peptide Recovery
Analysis of Serum Amyloid P Component in Plasma

Plasma was digested with trypsin or Trypsin/Lys-C overnight and analyzed with Xevo TQ-S (Waters).

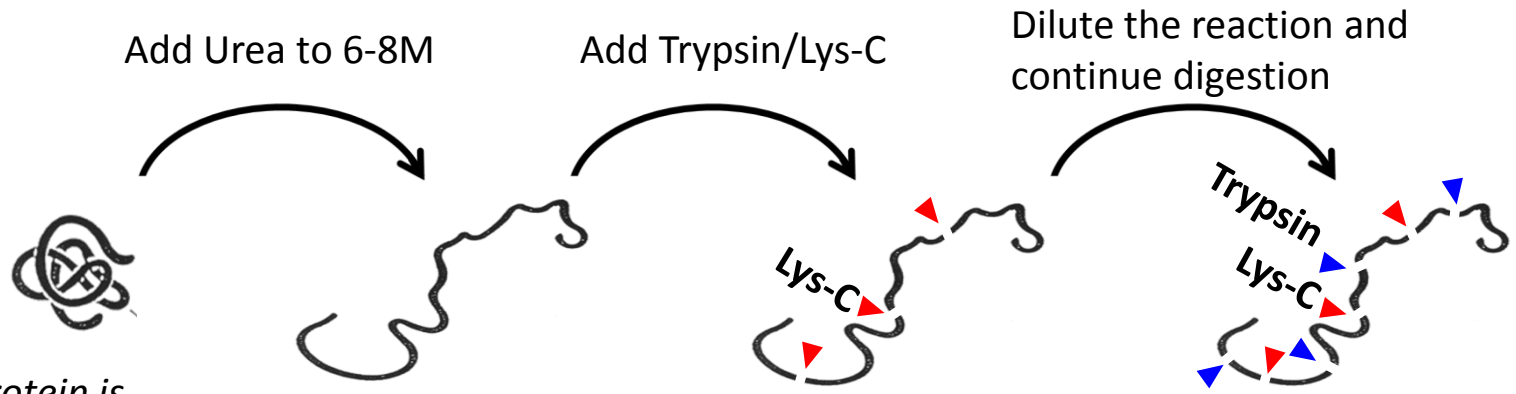


Trypsin/Lys-C improved SAP protein analysis.

Courtesy by M. Szapacs, GSK

Digestion of Proteolytically Resistant Proteins

Trypsin/Lys-C mix can digest tightly folded proteins, which are resistant to trypsin. <u>This digestion requires a specialized, two-step ("sequential") protocol, with the first step performed at strong denaturing conditions.</u>



Protein is resistant to trypsin due to tight conformation

Protein denatures. It is now amenable to proteolysis

Lys-C digests a protein onto relatively large fragments. Trypsin is inactivated.

Trypsin re-activates and completes digestion

Re-activated trypsin retains trypsin cleavage specificity

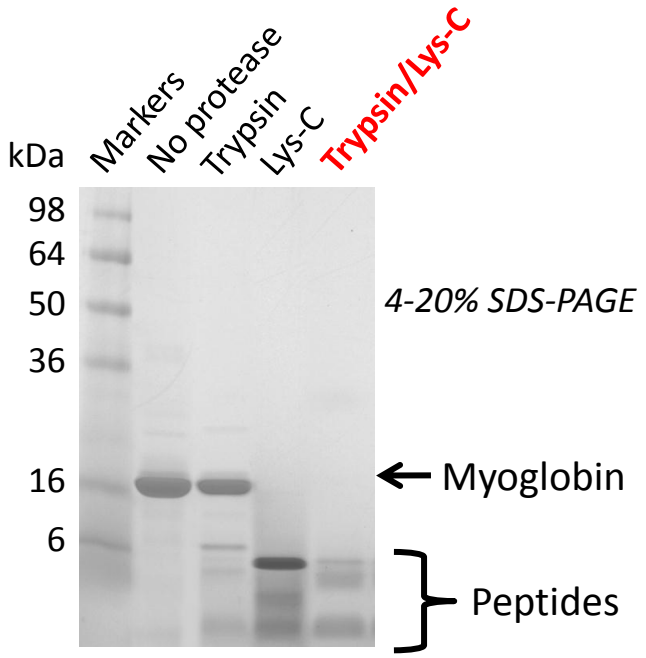
Ovalbumin peptides generated by re-activated trypsin

DSTTQINKVVR
GGLEPINFQTAADQAR
HIATNAVLFFGR
KIKVYLPR
LTEWTSSNVMEER
SALAMVYLGAK
VLVNAIVFK

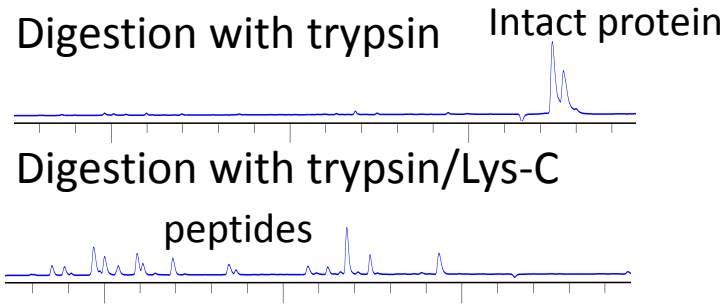
Improved Digestion of the Proteolytically Resistant Protein Myoglobin

Promega

Gel Analysis of Myoglobin Digests

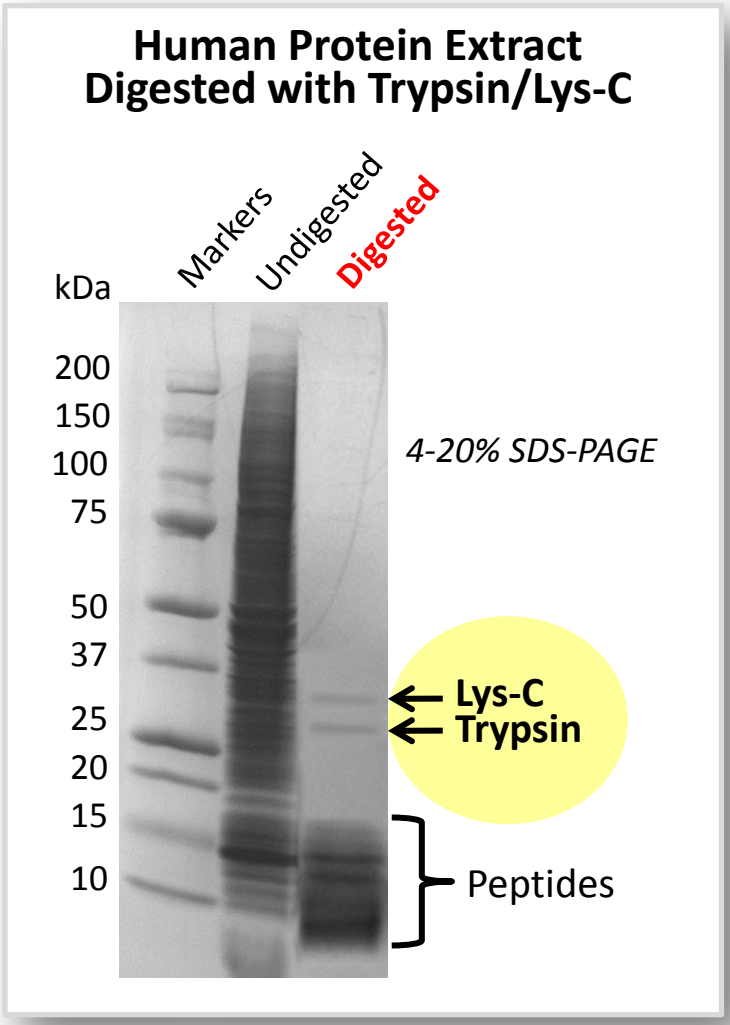


RP HPLC of Myoglobin Digests



Trypsin/rLys-C mix efficiently digests proteolytically resistant myoglobin.

Why Don't Trypsin and Lys-C Digest Each Other?



Trypsin is protected against autoproteolysis and Lys-C by ***methylation***.

The possible cause of Lys-C proteolytic resistance to trypsin is ***tight conformation***.

Applications

We recommend Trypsin/Lys-C for in-solution protein digestion.

In contrast to in-solution digestion, advantage of Trypsin/Lys-C might be minimal if any for in-gel protein digestion.

Protocols
Standard Overnight Digestion

Preparing the protein and trypsin/Lys-C solutions for digestion

- ✓ Reduce/alkylate protein as usual
- ✓ Dissolve Trypsin/Lys-C lyophilized mix in the supplied Resuspension buffer.
  We recommend dissolving to the final trypsin/Lys-C concentration of 0.5 µg/µl.

Digestion

Digest overnight at 37°C in 50 mM Tris-HCl*, pH 8 at 25:1 protein:(Trypsin/Lys-C) ratio.

**Other buffers have not been tested.*

Protocols
2-Step Digestion of Proteolytically Resistant Proteins

Preparing the protein and protease mix solutions

- ✓ Solubilize protein in 6-8 M Urea/50 mM Tris-HCl, pH 8. Reduce/alkylate as usual but do not exceed 37°C temperature during reducing step*

  **Higher temperature will induce carbamylation.*

- ✓ Dissolve Trypsin/Lys-C lyophilized mix in the supplied 'Resuspension buffer' (50 mM acetic acid)

  We recommend dissolving the final trypsin/Lys-C concentration of 0.5 µg/uµl.

Two-step digestion

Step 1. Add Trypsin/Lys-C solution to protein solution at 25:1 protein:(Trypsin/Lys-C) ratio. Incubate 3-4h at 37°C.

Step 2. Dilute the reaction 4-fold with 50 mM Tris-HCl, pH 8. Continue digestion overnight at 37°C.

Trypsin/Lys-C Composition and Format
Multiple Sizes Available to Match Your Throughput

Promega

Composition

✓*The highest quality trypsin, Trypsin Gold, is used in Trypsin/Lys-C.*
✓*As a source of Lys-C we use highly robust and cleavage specific recombinant Lys-C.*

Format

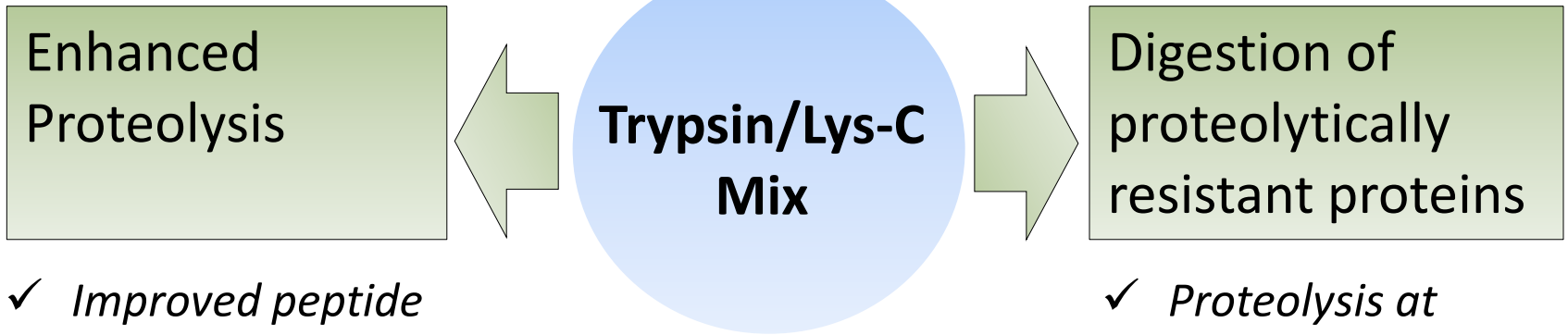
Trypsin/Lys-C Mix is provided in a lyophilized form

V5071 - single 20 µg vial
V5072 - single 100 µg vial
V5073 - 5x20 µg kit

All the products are supplied with a vial of Resuspension buffer (500 µl).

Trypsin/Lys-C Mix Advantage

Better Proteoloysis = Improved Mass Spec Analysis



- ✓ *Improved peptide and protein identification*
- ✓ *Accurate protein quantitation*
- ✓ *Reproducible analysis*

- ✓ *Proteolysis at strong denaturing conditions*

Contact Information

Sergei Saveliev, R&D Senior Scientist
sergei.saveliev@promega.com

Thank you for your interest in our new products!