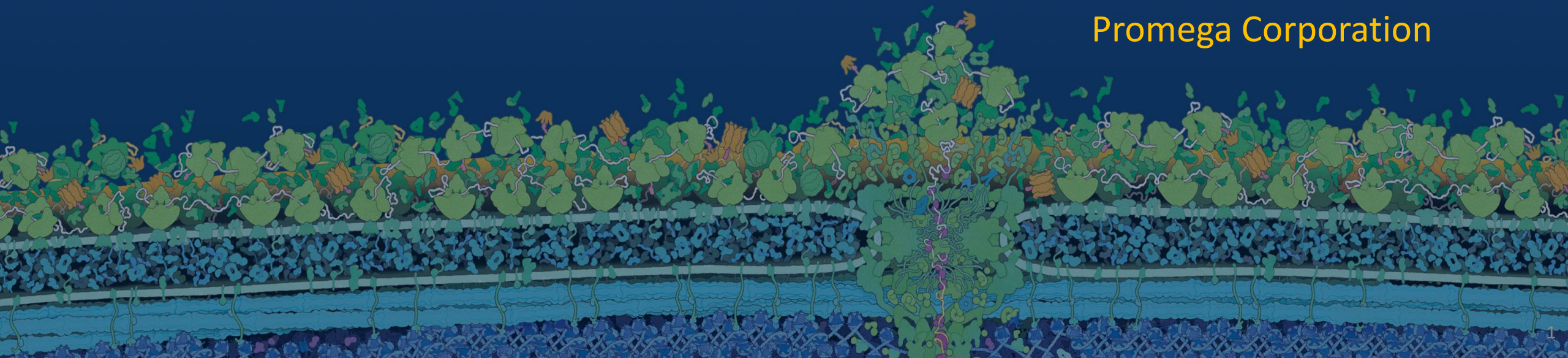
ProAlanase: A Proline- and Alanine-Specific Protease for Proteomics

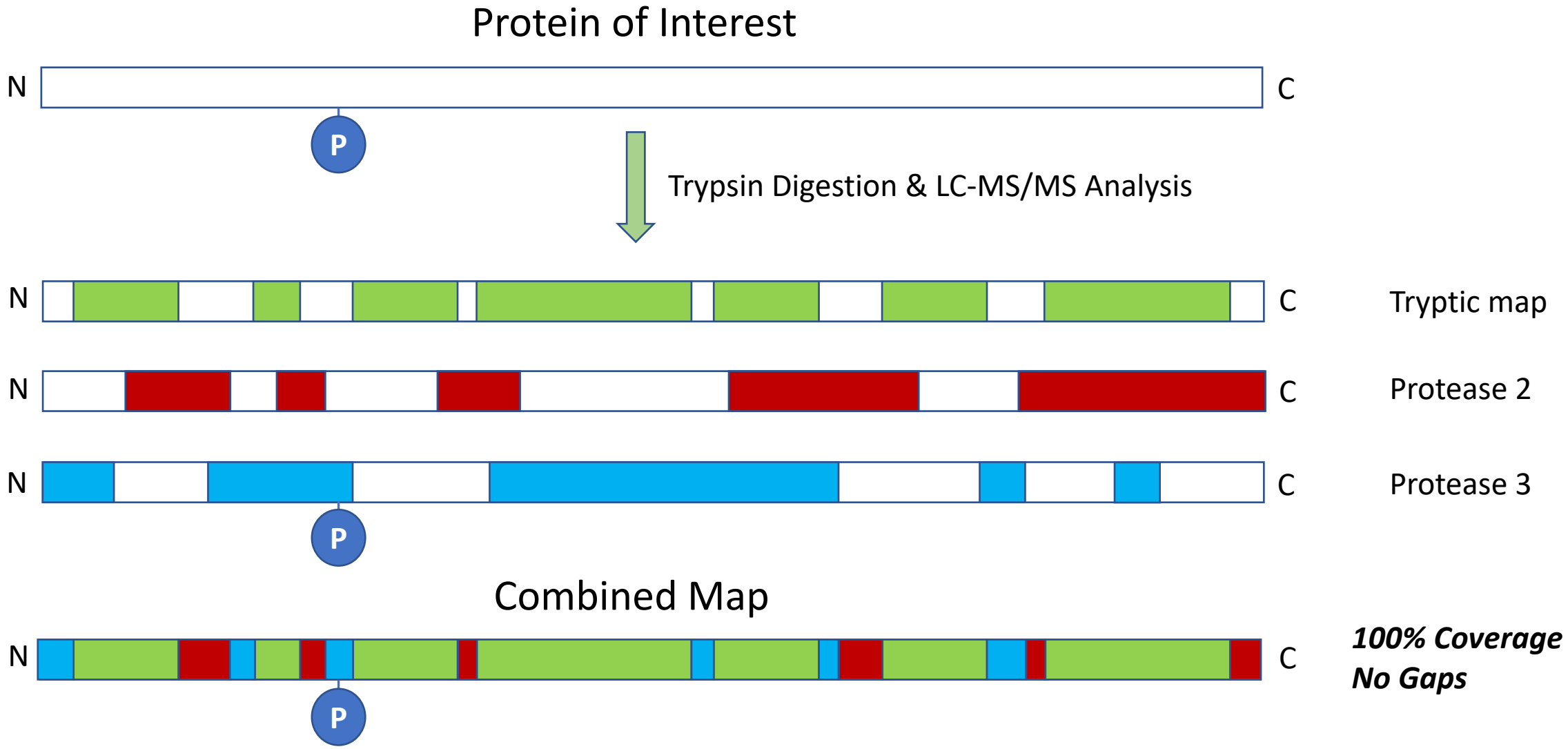
Chris Hosfield, PhD

Senior Research Scientist

Promega Corporation



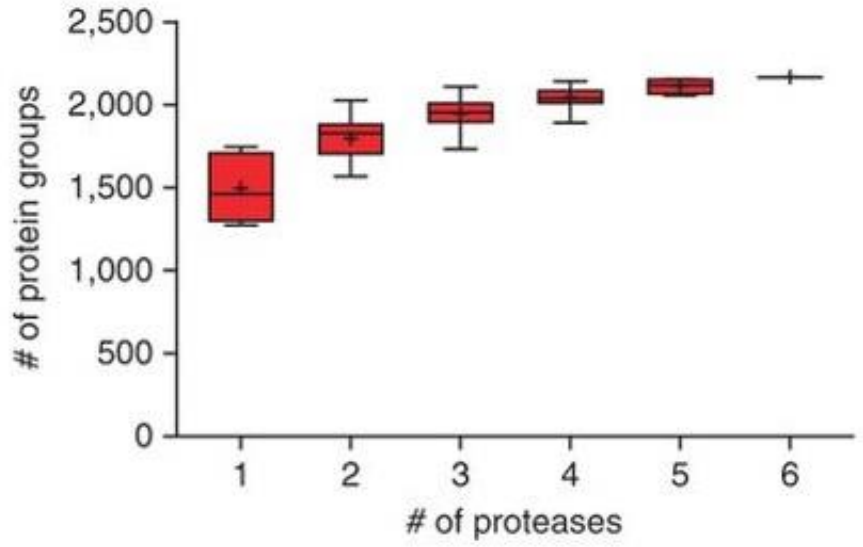
Trypsin is Great: But Rarely Gives the Full Picture



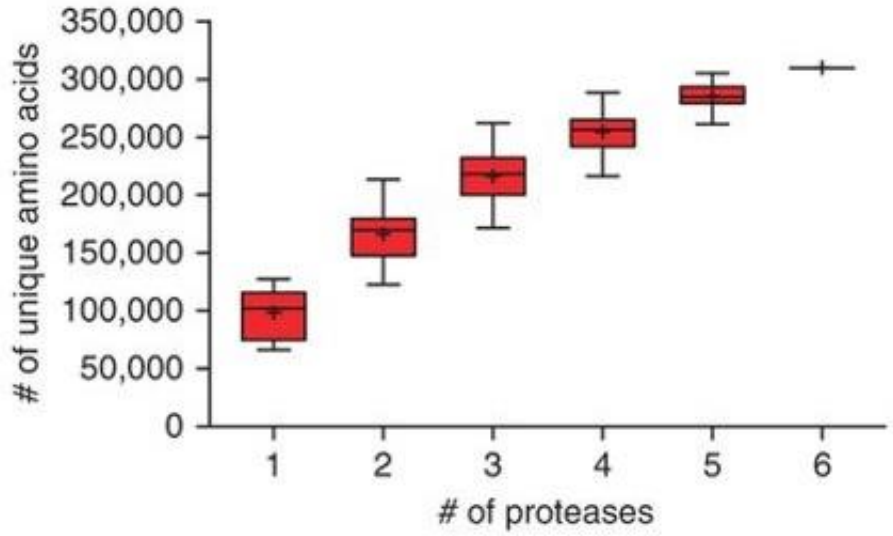
100% Coverage
No Gaps

Using Multiple Proteases Improves Data Quality

Protein Identifications



Amino Acid Coverage



E. Coli lysate

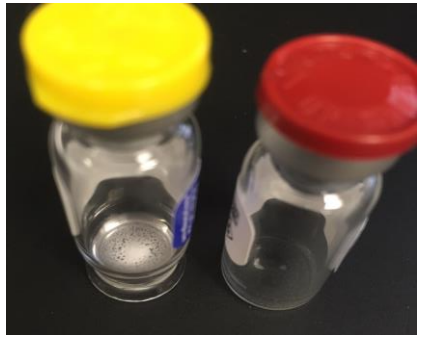
Giansanti et al. (2016)
Nature Protocols

Recent Addition: Recombinant Asp-N (rAsp-N)

- High performance – efficient digestion in one hour
- His-tag for easy removal
- Improved format
  - New formulation enhances product stability and consistency of use
  - V-shaped vial and larger size (10 µg)

NEW

V-shape Flat-Bottom



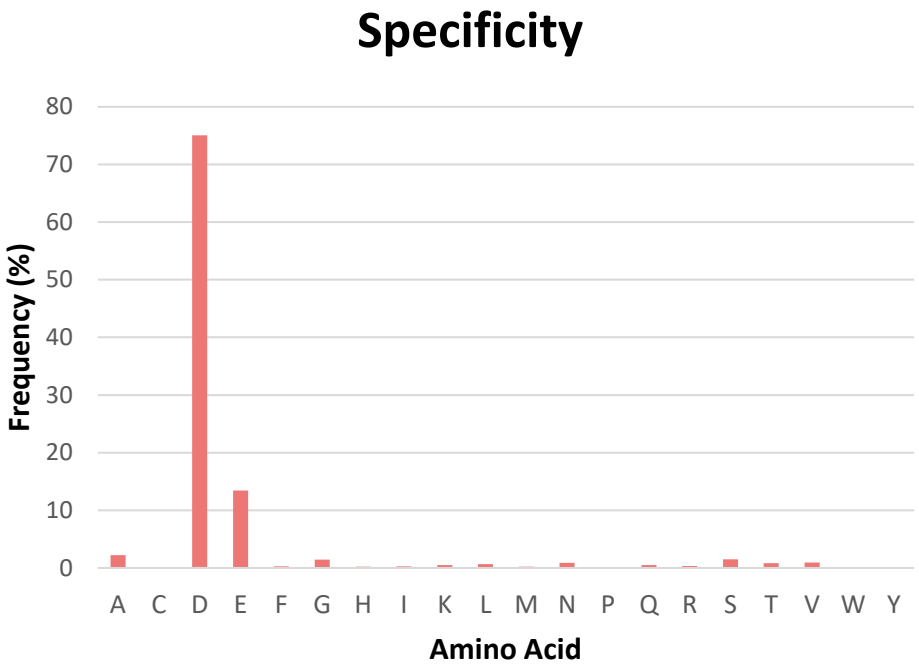
Enhanced formulation

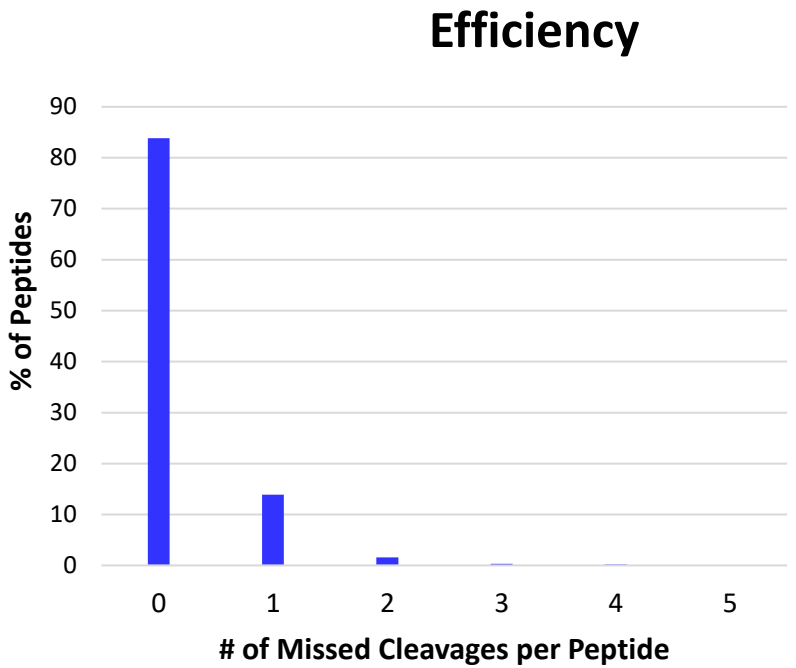
Better price

~1/2 the cost of native per µg

rAsp-N is a High-Performance Protease

- Yeast extract was digested for 1 hour at 37°C in ~1.5M urea at a 1:50 ratio

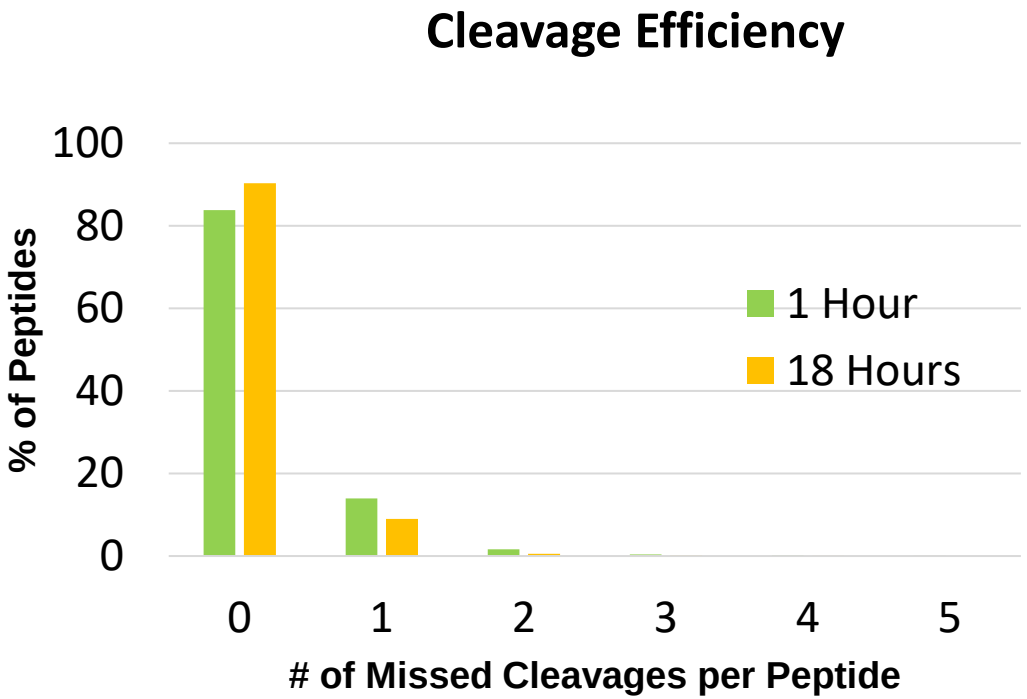


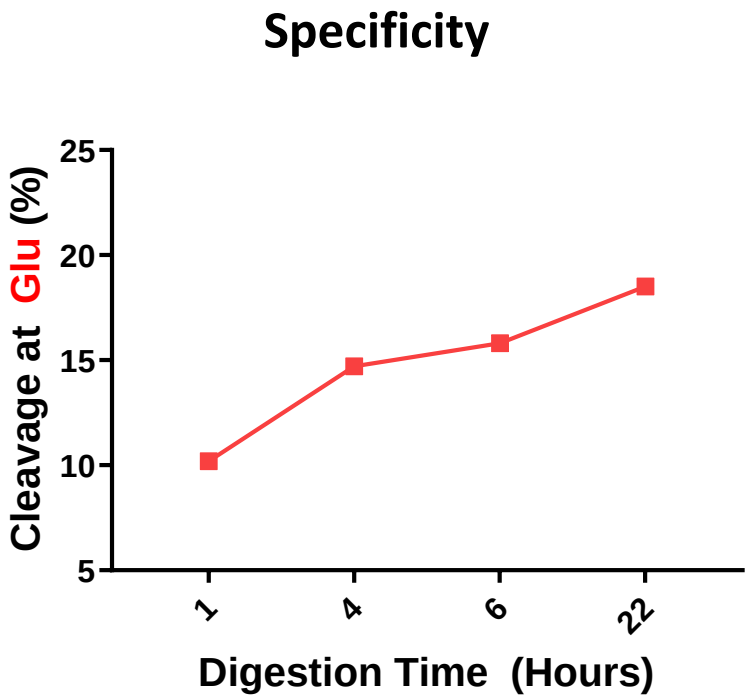


- Primarily cleavage at Asp
- Secondary cleavage at Glu

- Cleavage at Asp is ~85% complete

What Happens in Longer Digests?



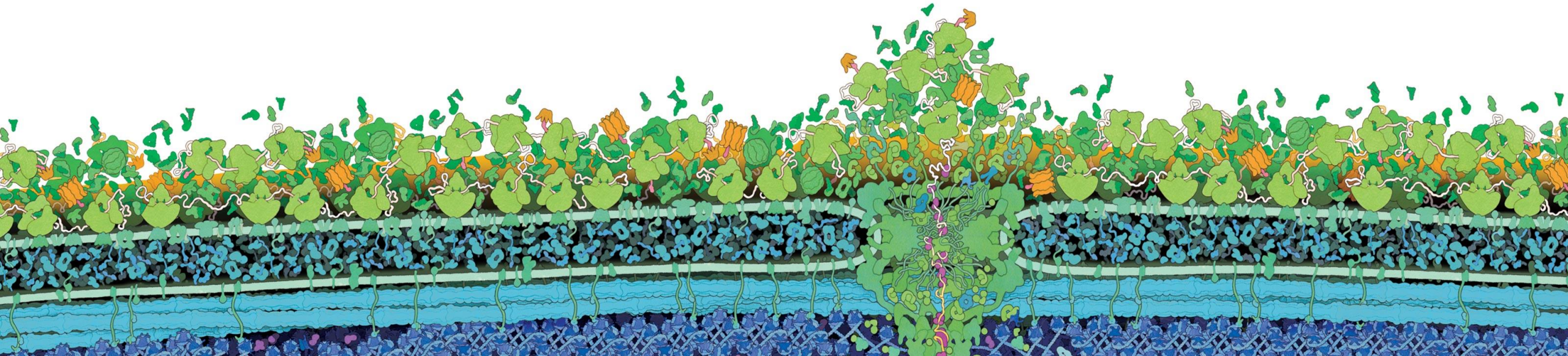


Cleavage efficiency at Aspartic acid improves slightly
Increased # of cleavages at Glutamic acid reduces specificity

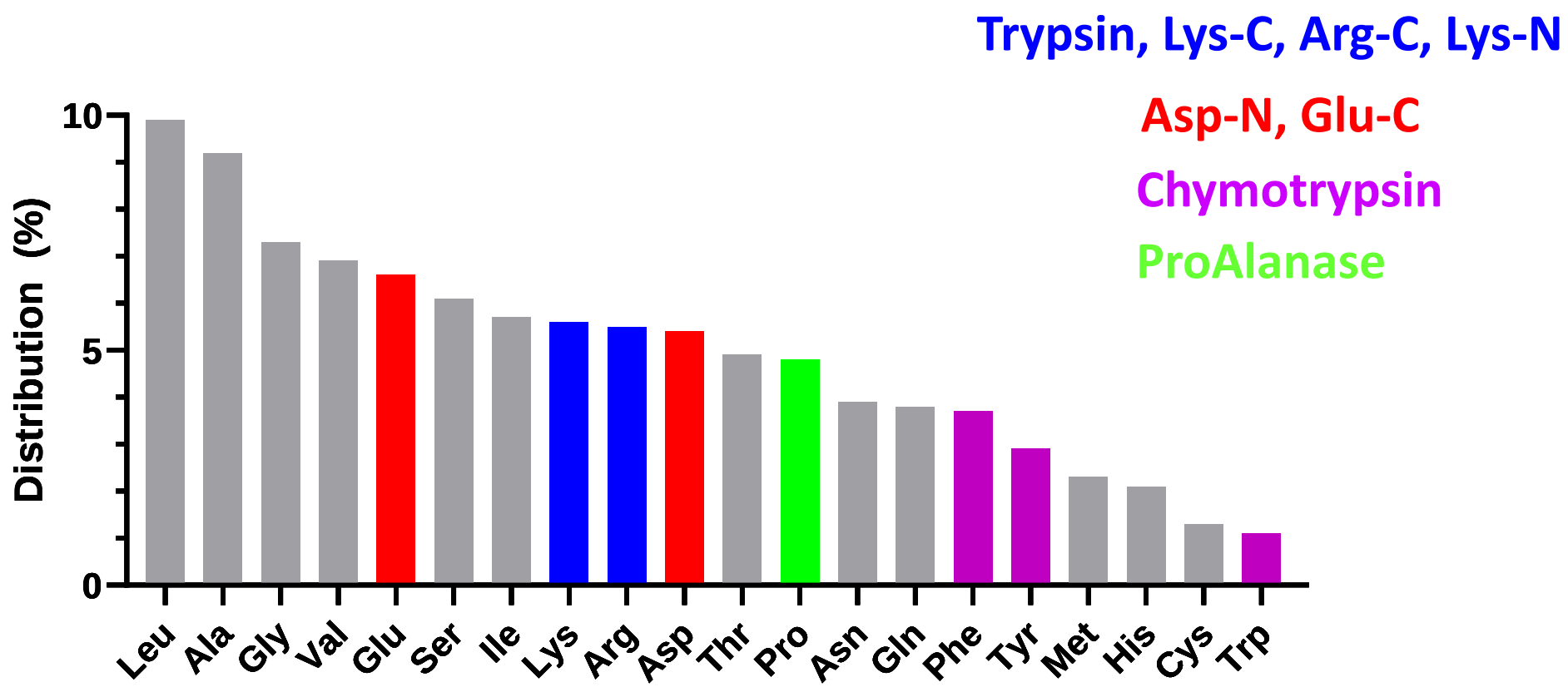
Promega

ProAlanase

New Protease with Specificity for Proline and Alanine



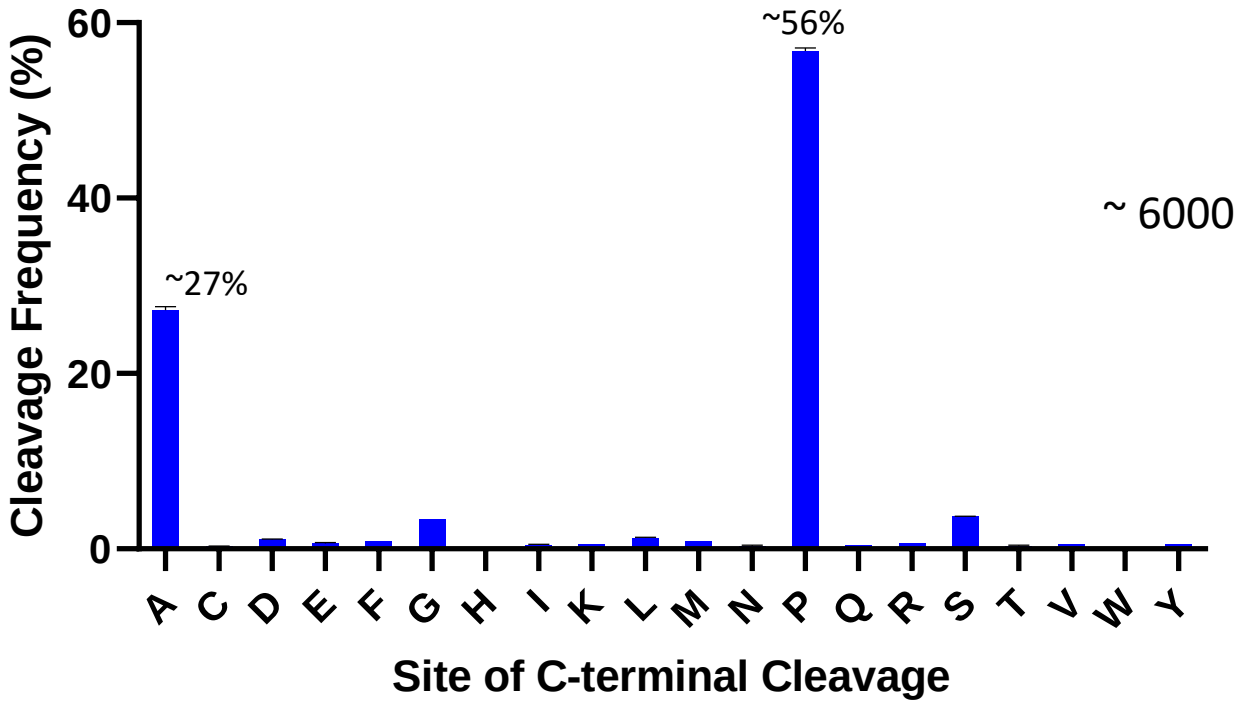
Current Proteases do not Address the Full Proteome



Source: UniProt database, Sept 2019

ProAlanase Cleaves Primarily at Proline and Alanine

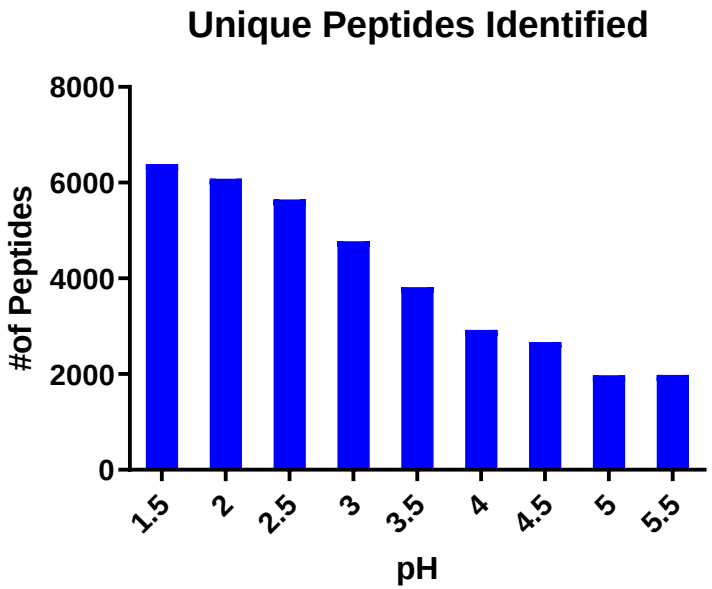
- ProAlanase is a serine protease from *Aspergillus niger* active at low pH
- Human K562 extract was digested with ProAlanase at pH 1.5 for 2 hours at 37°C at a 1:100 E:S ratio.
- Data collected with a Q Exactive Plus.
- Data were searched with Byonic (Protein Metrics) with no enzyme specified.

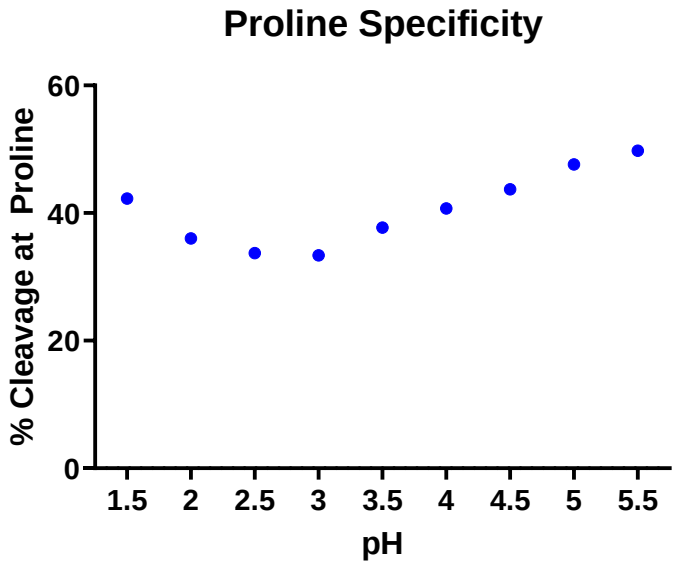


~ 6000 unique peptides

ProAlanase Performance is Optimal around pH 1.5

- Human K562 extract digested with ProAlanase *at various pH values* for 2 hours at 37°C at a 1:50 E:S ratio.

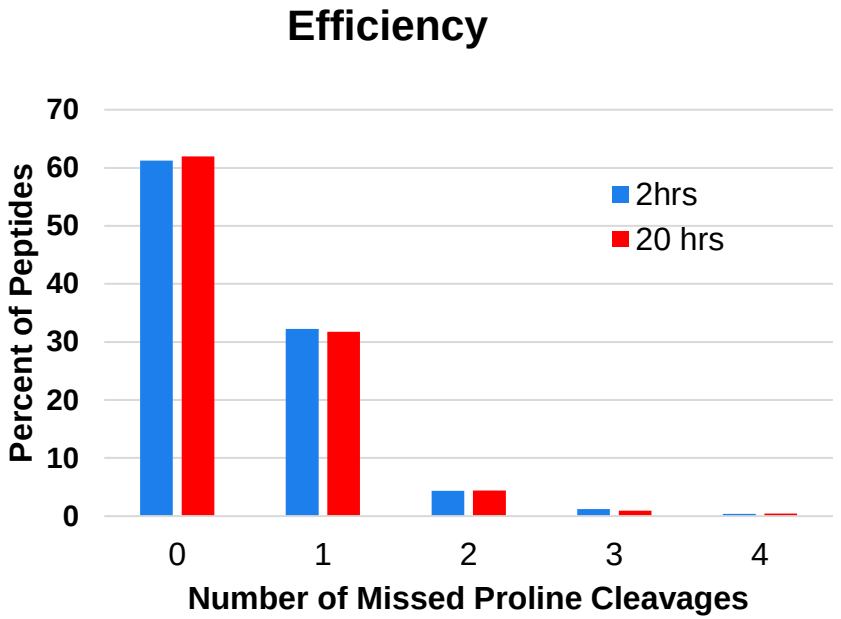


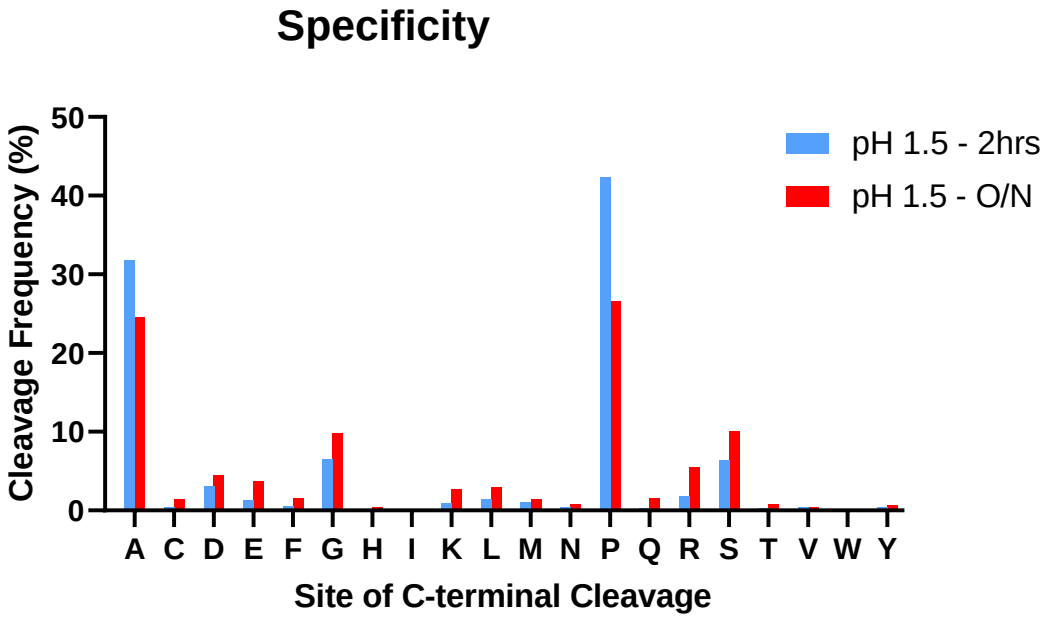


Best combination of specificity and peptide IDs observed at pH 1.5

ProAlanase Performance is Optimal with Short Digestions

- Human K562 extract digested with ProAlanase at pH 1.5 *for 2 hours or overnight* at 37°C at a 1:50 E:S ratio.

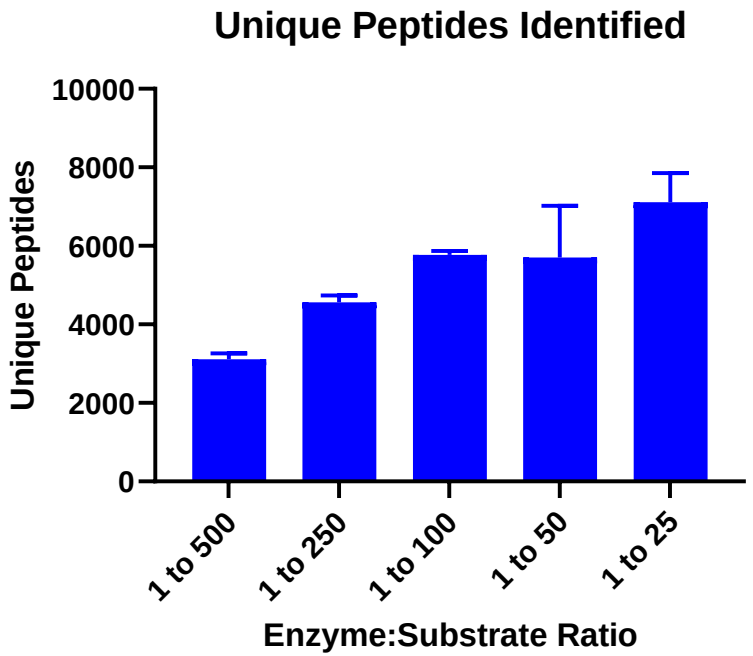


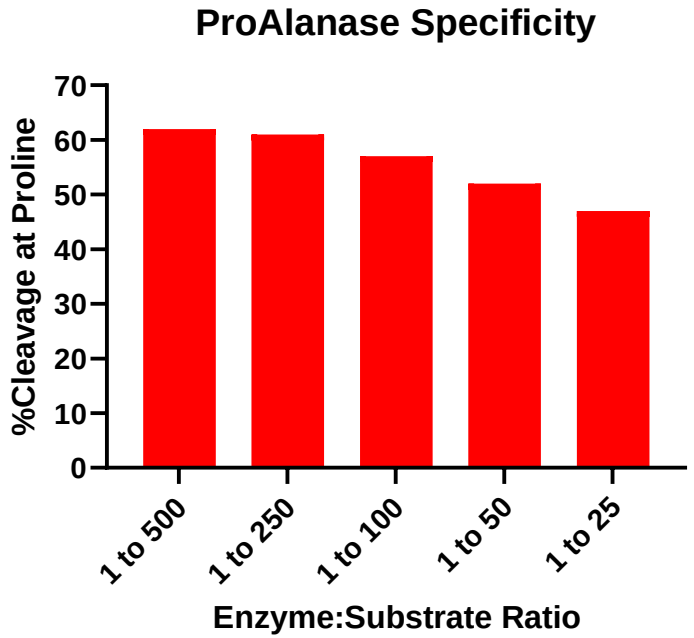


Increasing digestion time beyond 2 hours does not increase efficiency but reduces specificity

Changing ProAlanase Amount Affects Performance

- Human K562 extract digested with ProAlanase *at various E:S ratios* for 2 hours at 37°C at pH 1.5.





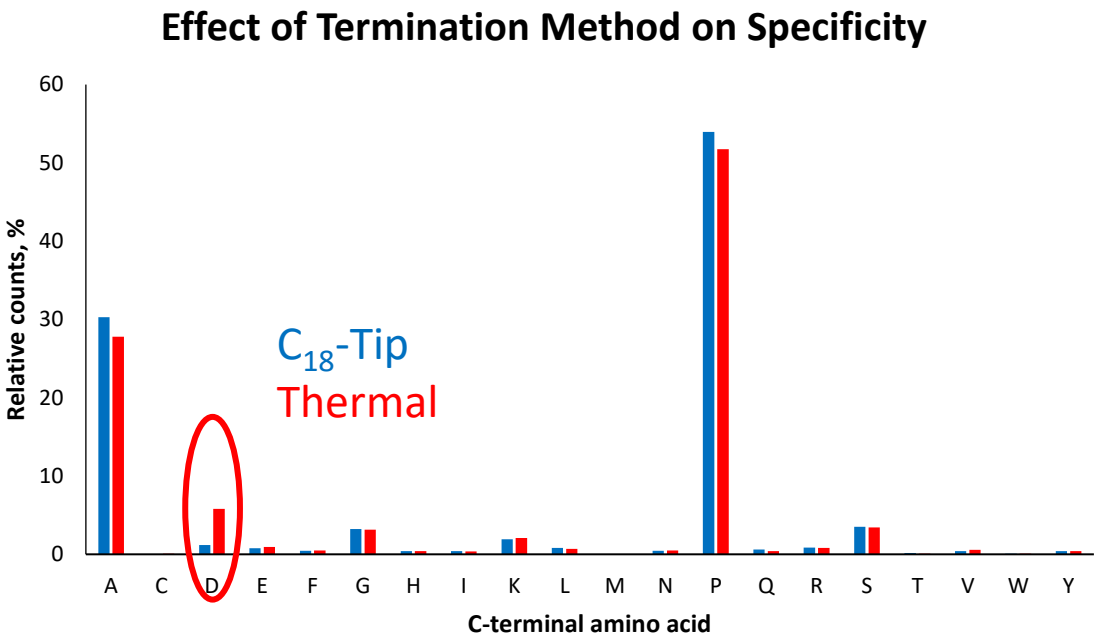
Increasing ProAlanase increases peptides IDs but reduces specificity

How to Terminate Digestions with ProAlanase?

- Acidification with TFA or Formic Acid does not inhibit ProAlanase
  - Over-digestion of samples will occur unless reaction is terminated

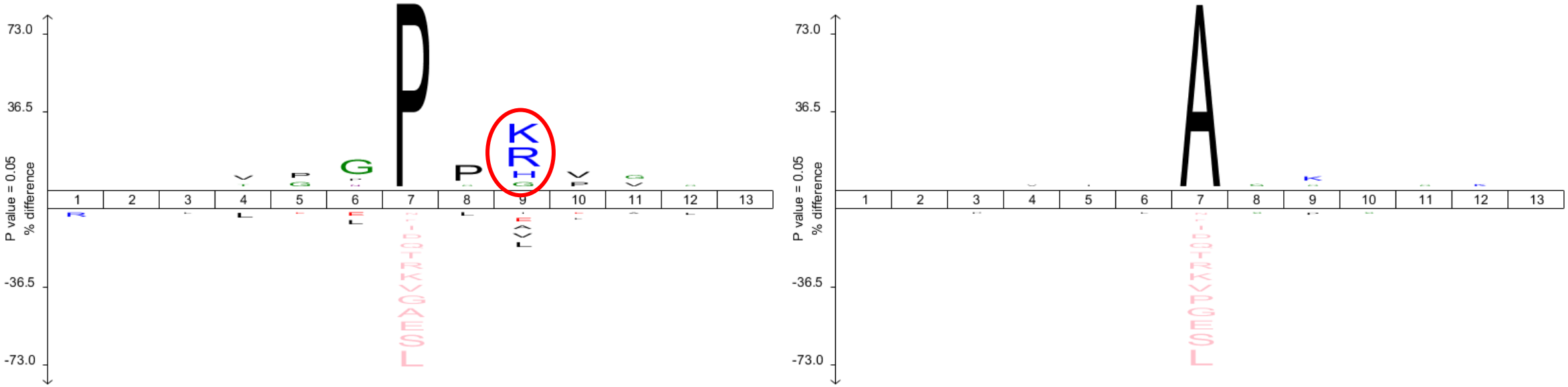
Option 1: Heat 10 minutes at 90-95°C

Option 2: C_{18}-based cleanup (e.g. C_{18} Tips)



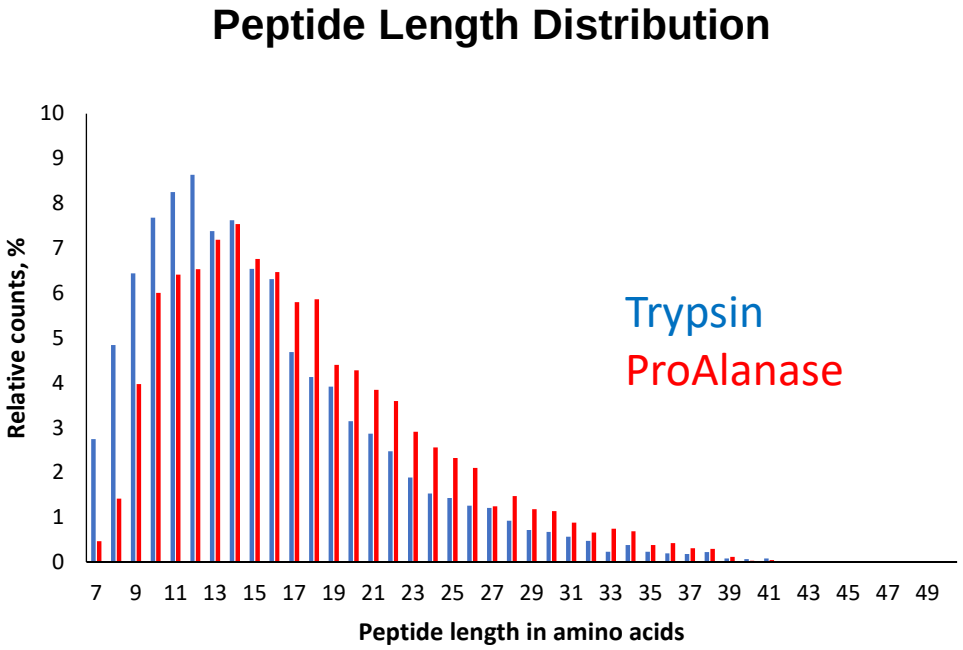
Termination Method	Ease of Use	Fragmentation?
Heat-based	Easy	Yes: D-P bonds
C_{18} cleanup	Cumbersome (multiple samples)	No

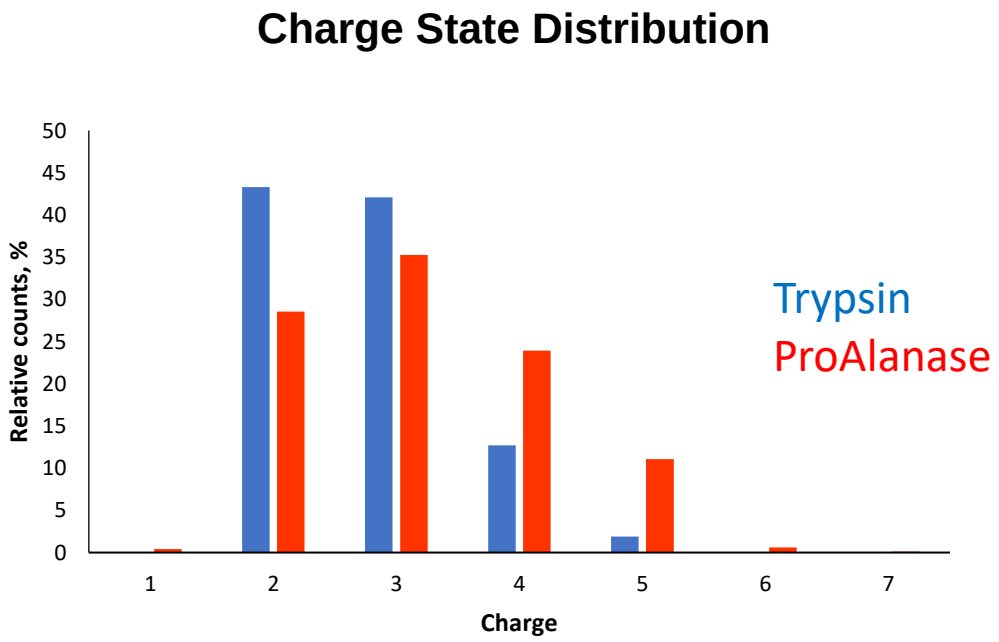
IceLogo Analysis of ProAlanase Cleavage Patterns



- Missed proline cleavage typically due to basic residue in P2’ position.
- No clear patterns on missed cleavage at alanine

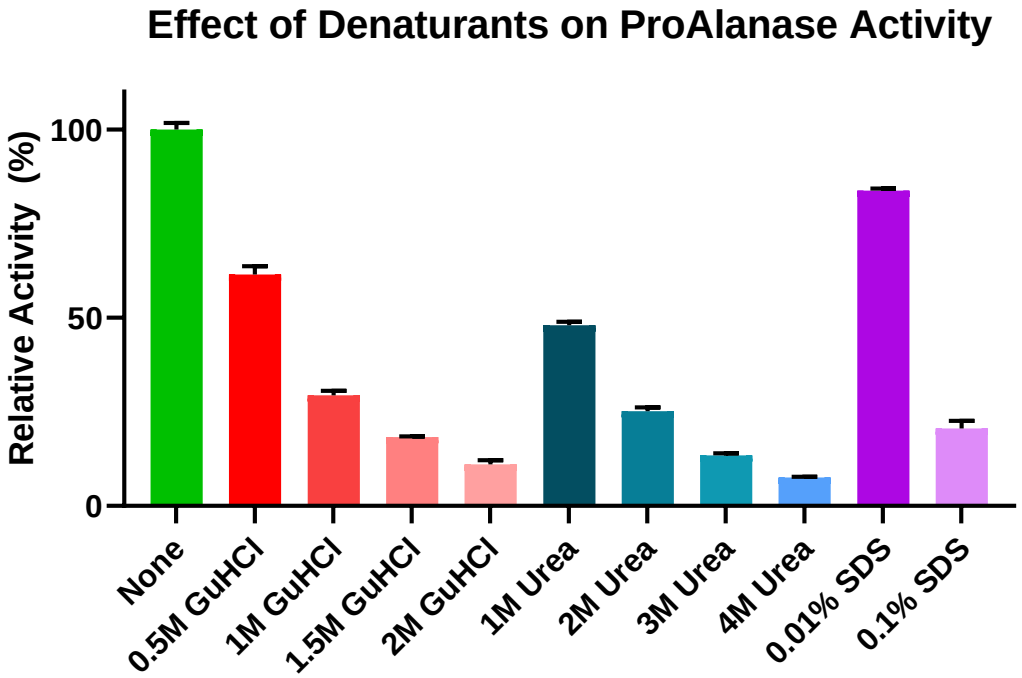
Characteristics of ProAlanase-Derived Peptides



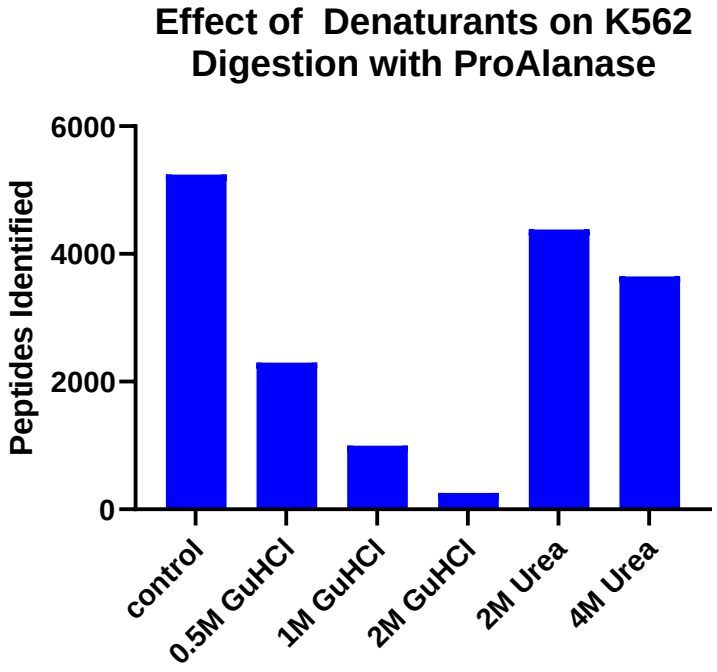


- Longer peptides than produced from trypsin
- More peptides at higher charge states than produced from trypsin

Effect of Denaturants on ProAlanase Activity



Synthetic Peptide Fluorescent Assay

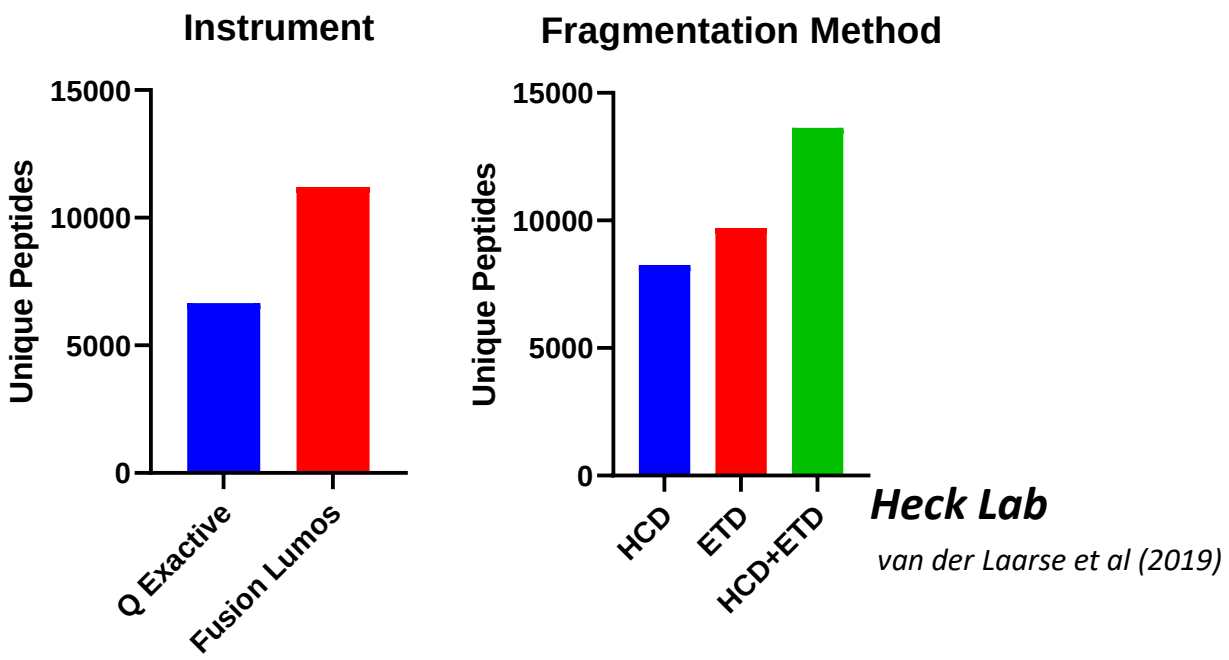


K562 Digest & LC-MS/MS

- 2-4M urea preferred if denaturant is needed
- *Additional denaturants may not be needed at pH 1.5!

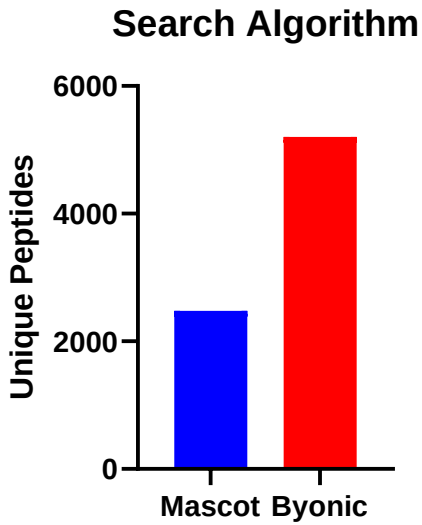
MS Instrument and Software Affect Results

Instrumentation



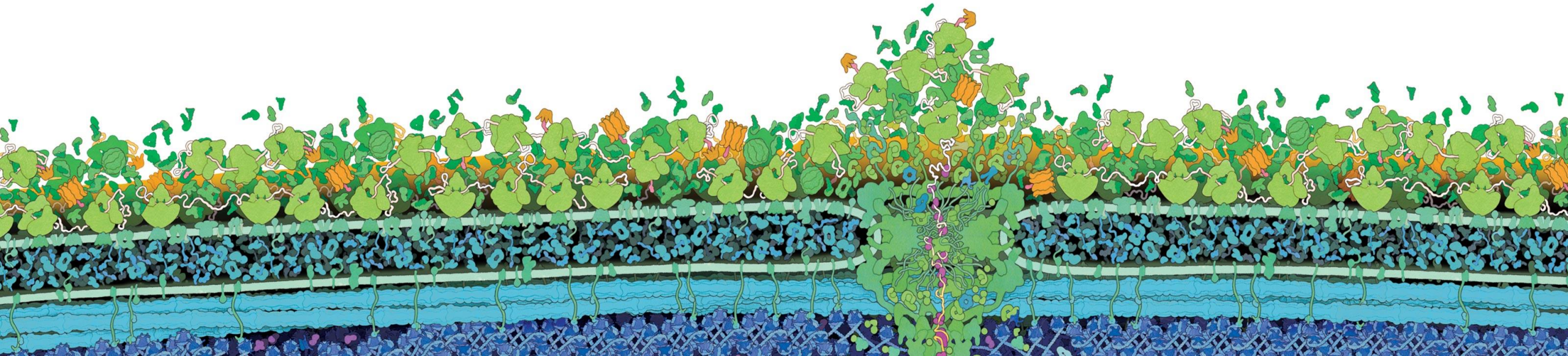
van der Laarse et al (2019)

Software



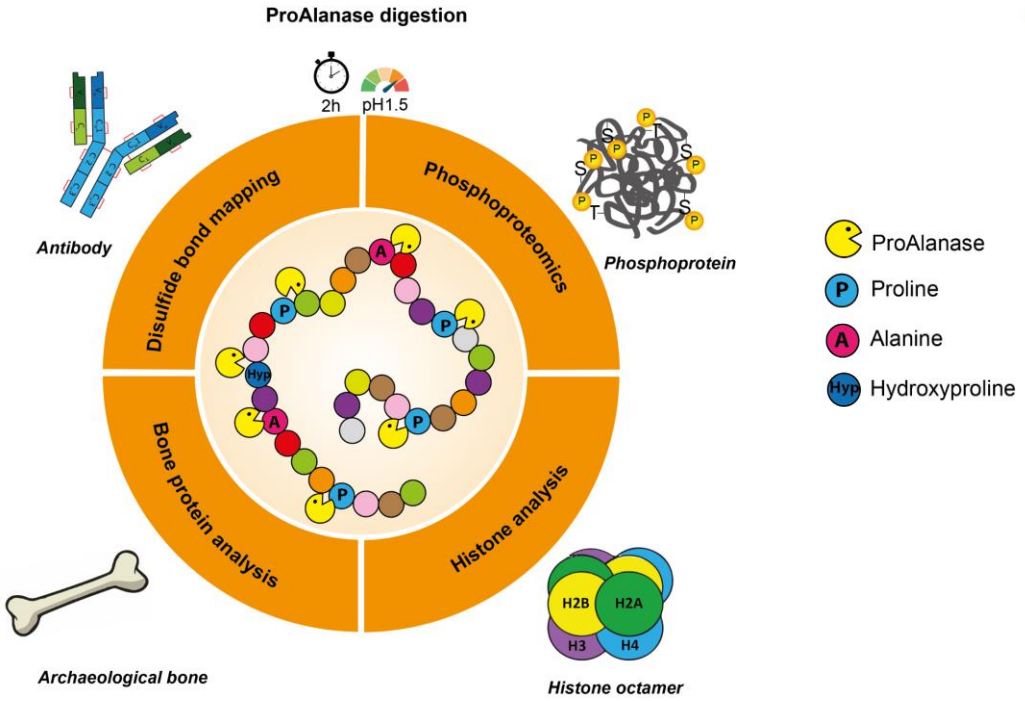
Optimize data collection and search strategies to increase peptide IDs

Applications



Applications of ProAlanase

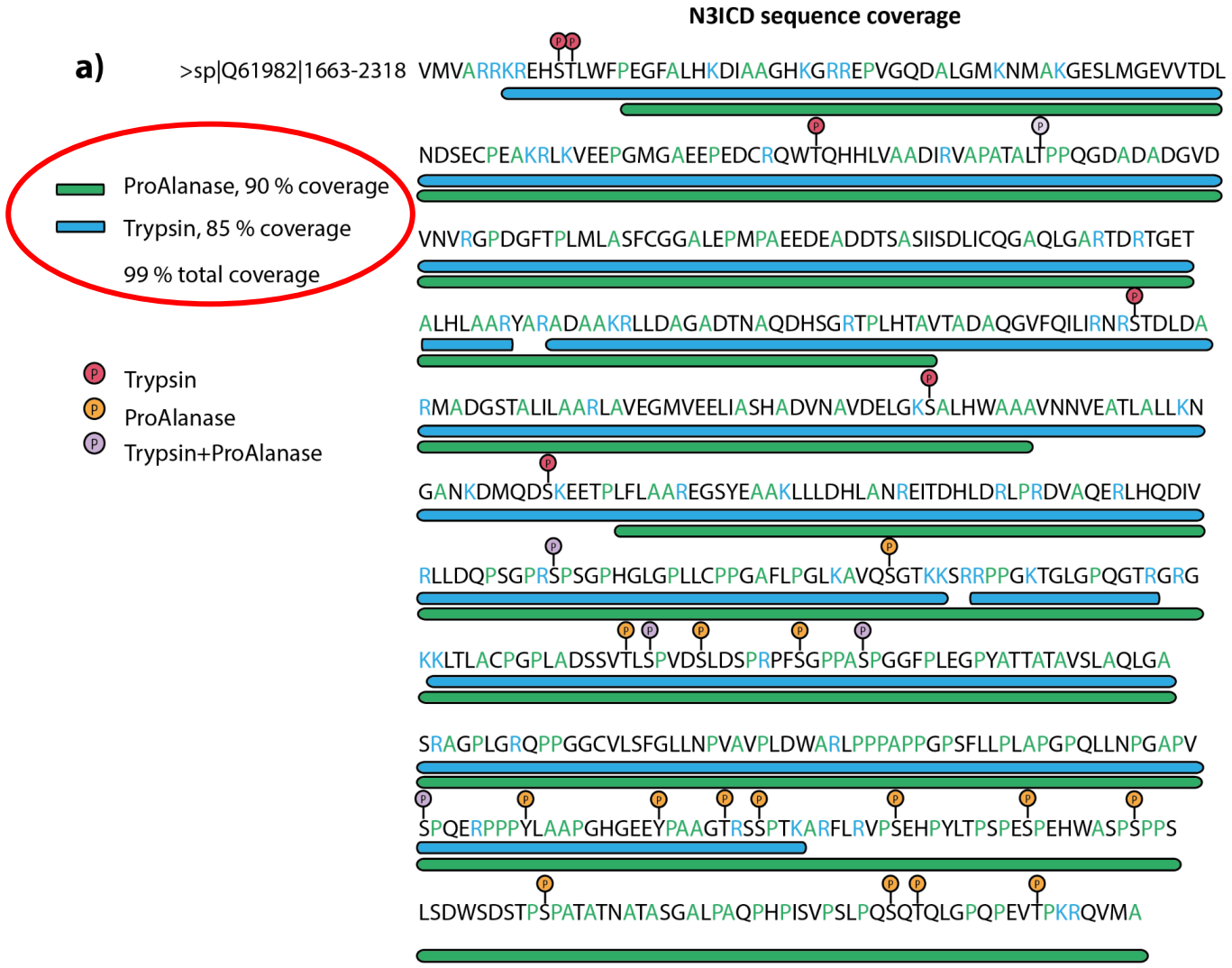
- Proteomics
- Protein characterization / Peptide mapping
- Disulfide bond mapping
- *De novo* sequencing
- Histone characterization
- HDX-MS



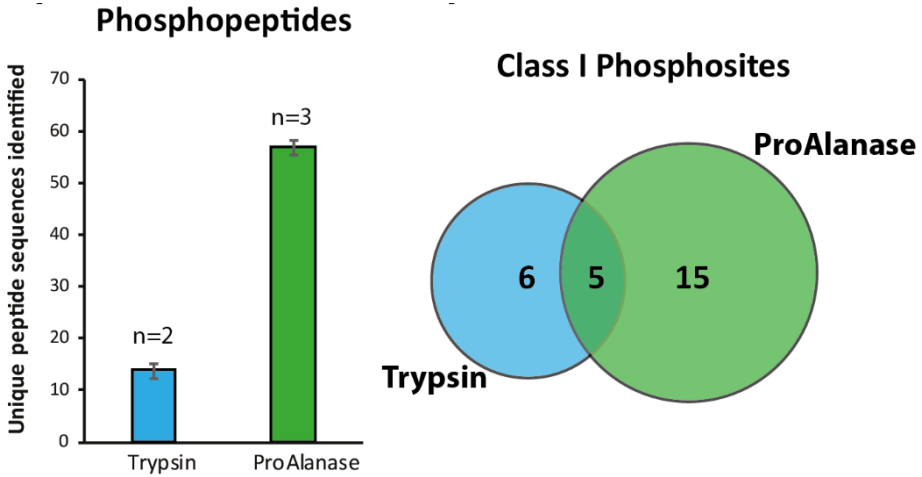
New: Publication in Press

Samodova, D. *et al.* (2020) ProAlanase is an effective alternative to trypsin for proteomics applications and disulfide bond mapping.

Increase Sequence Coverage and Phosphosite Mapping

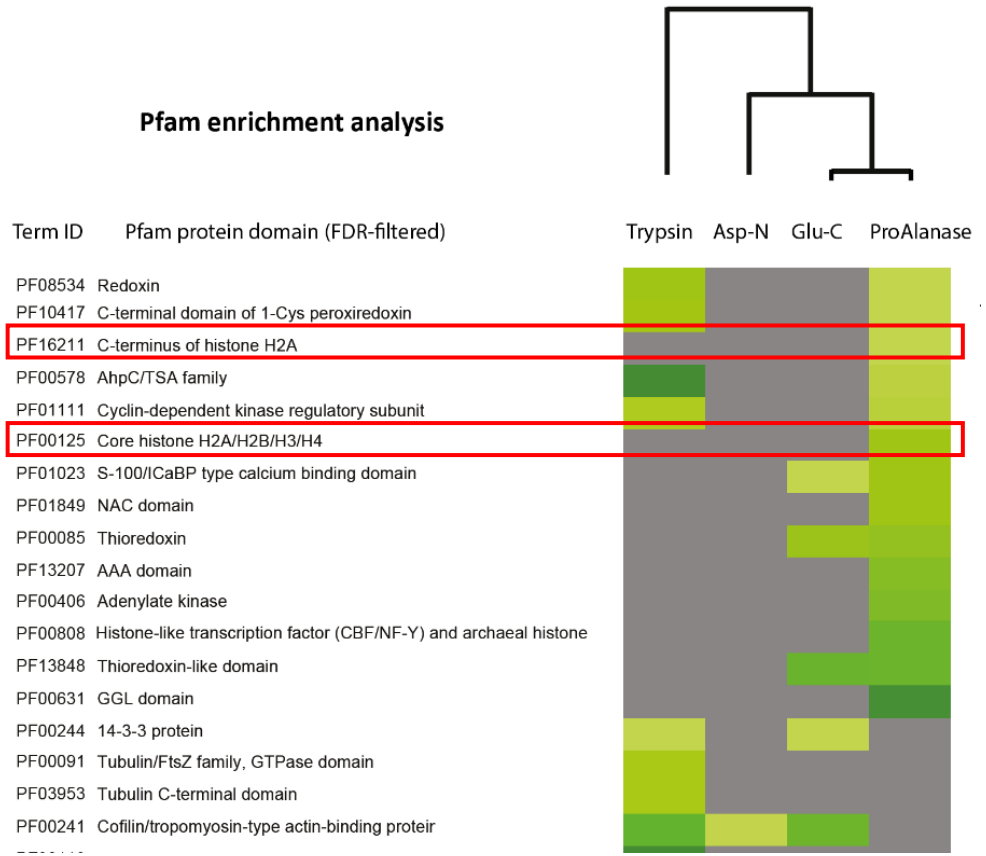


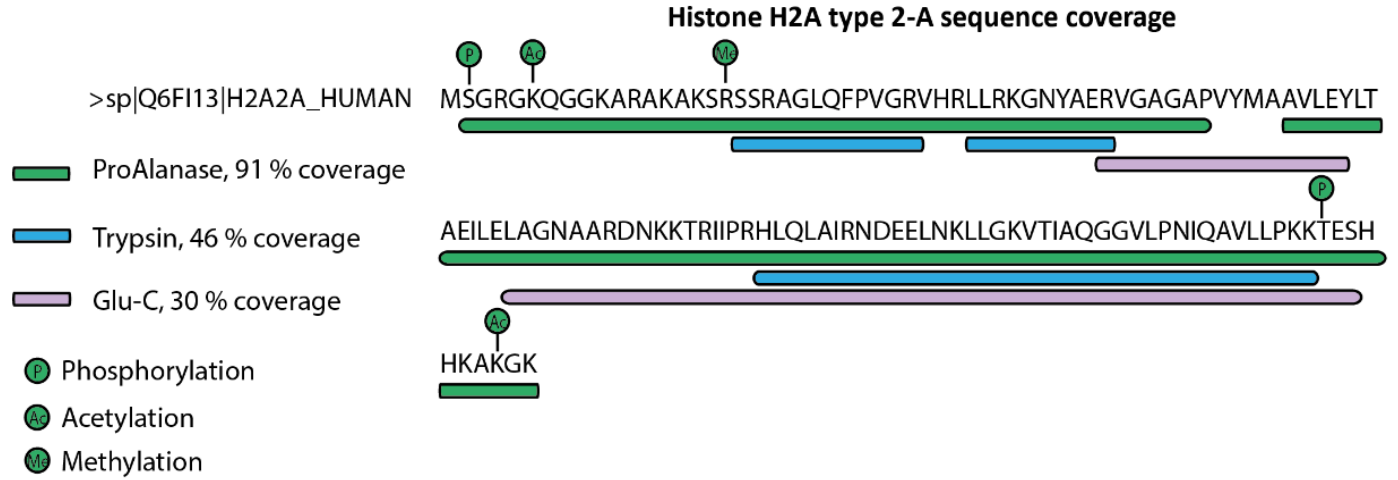
Data generated from in-gel digestion after I.P.



Increased sequence coverage
Increased phosphosite IDs

Histone Characterization





- ProAlanase significantly improves coverage of Histones compared with other proteases
- Allows for characterization of important Histone PTMs

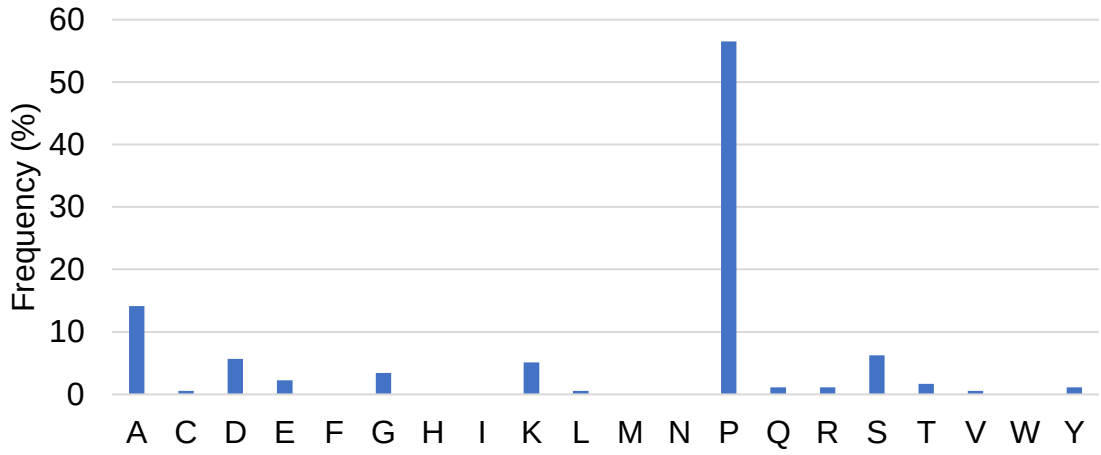
Peptide Mapping of Biotherapeutic Proteins

- NISTmAb was digested with ProAlanase *at various E:S Ratios* for 2 or 18 hours at 37°C

Sequence Coverage

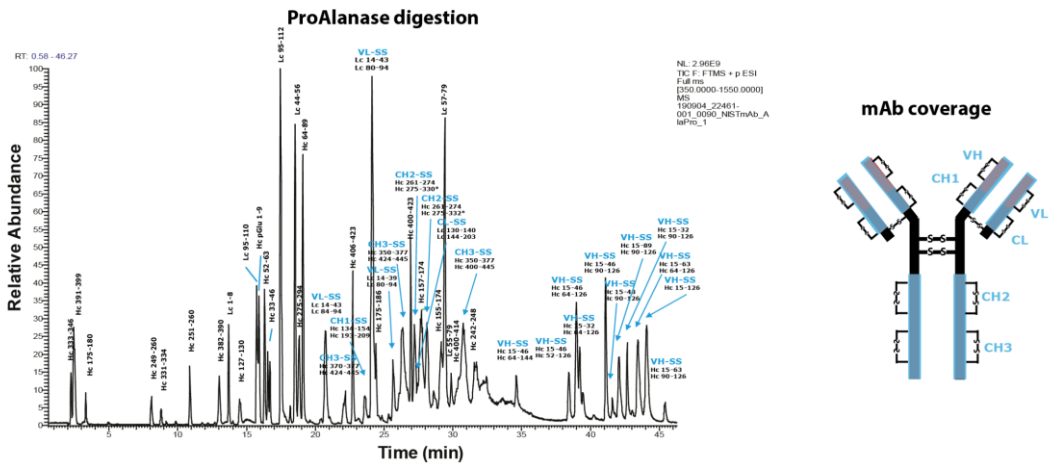
	1:250 E:S		1:100 E:S		1:50 E:S	
	1 Hr	18 Hrs	1 Hr	18 Hrs	1 Hr	18 Hrs
Heavy Chain	97	79	98	77	95	68
Light Chain	97	94	97	90	97	73

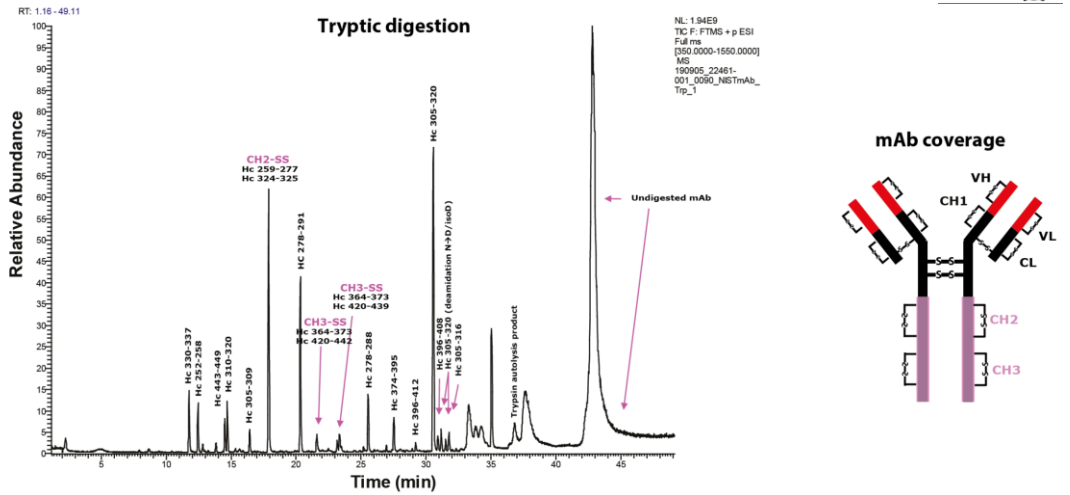
Cleavage Specificity



- High coverage and high specificity obtained
- Short digestion times and low pH help minimize artifacts

Disulfide Bond Mapping



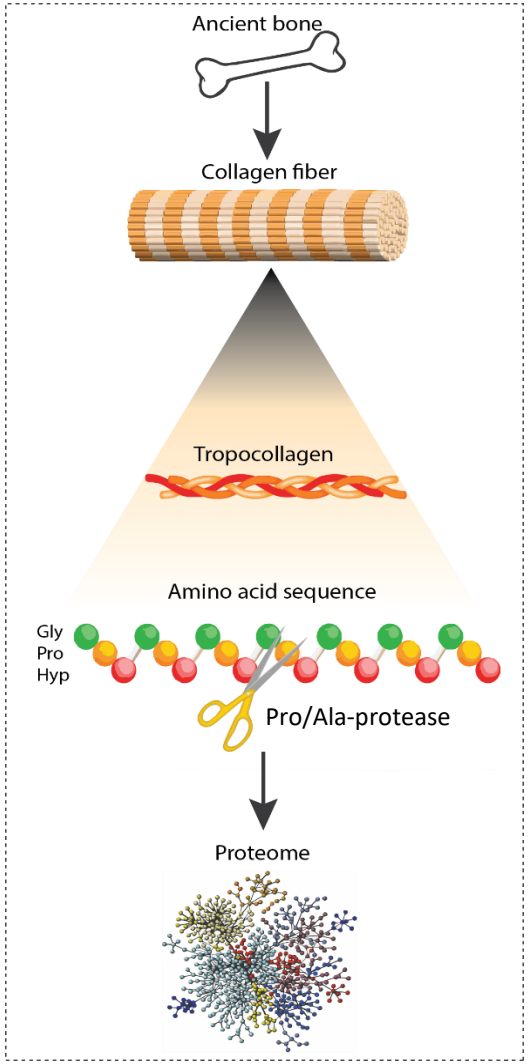


- Efficient digestion at pH 1.5
- Assignment of most S-S bonds

- Poor digestion at pH 8.0
- Assignment of few S-S bonds

- Low pH environment minimizes disulfide bond scrambling and artificial PTMs like deamidation
- Acidic pH acts as an effective denaturant of NISTmAb IgG allowing efficient digestion
- Assignment of all non-hinge disulfide bonds

ProAlanase and Paleoproteomics



Collagen

- Most abundant protein in bone **(~ 90%)**
- Well conserved thus not a good phylogenetic marker
- Supresses other protein identification.
- Repetitive sequence motif: Gly-**Pro**-X & Gly-X-**Hyp**

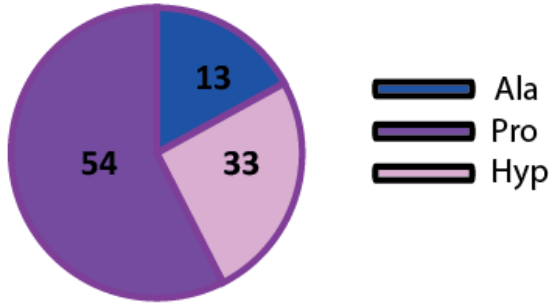
Aim: Chop down collagen with ProAlanase and improve identification of other bone proteins.

Non-collagenous bone proteins can be used for:

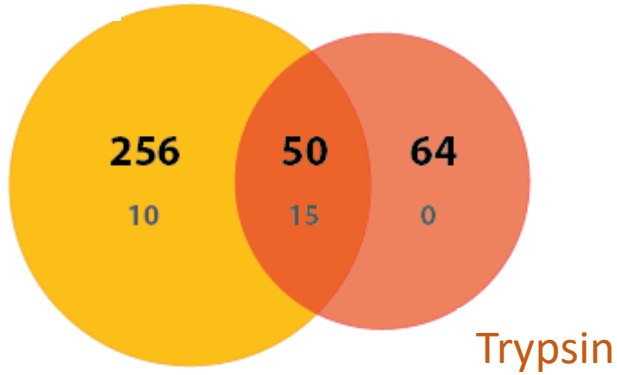
- Reliable species identification
- Phylogenetic placement
- Disease identification

Paleoproteomics of the Woolly Mammoth

ProAlanase Cleavage Sites in Bone



ProAlanase

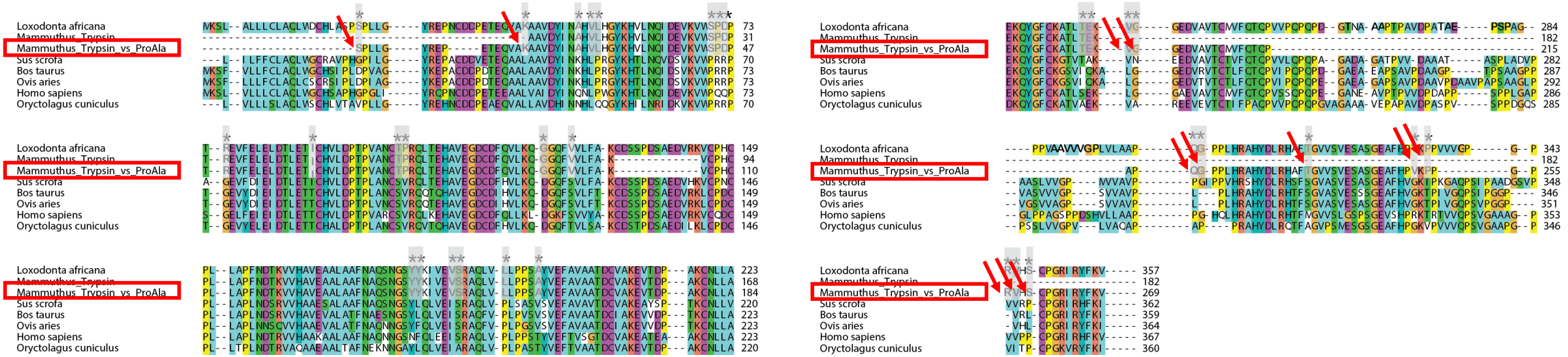


Protein groups IDd from mammoth bone

- ~3x more protein groups are identified in ProAlanase-digested sample
- 4x > complementary **non-collagenous** proteins (246), compared to trypsin (64)
- 10 complementary **collagen isoforms** identified with ProAlanase and 0 – with trypsin.

Improved Sequence Coverage of Mammoth Fetuin

Fetuin-A sequence coverage across different species



- Combining peptides from trypsin and ProAlanase increases coverage of species-specific proteins.
- ProAlanase should be an effective tool for paleoproteomic analysis.

ProAlanase Now Available

Product	Amount	Concentration
ProAlanase	5 µg	0.2 µg/µL
ProAlanase Plus	15 µg	0.5 µg/µL

Ordering:
To purchase ProAlanase through our Early Access program, please contact your Promega sales representative directly for a quote. Alternatively, please email our early access team at CAS@Promega.com

Technical Support:
For technical questions, please contact Chris Hosfield, Senior R&D Scientist chris.hosfield@promega.com

Acknowledgements

University of Copenhagen

Jesper Olsen

Diana Samodova

Christian Kelstrup

Giulia Franciosa

Enrico Cappellini

Novo Nordisk A/S

Christian Cramer

Sapienza University of Rome

Maria Giuli

Promega Corporation

Mike Rosenblatt

Alpha Testers

Francis Crick Institute

Karolinska Institute

Lunenfeld-Tanenbaum Research Institute

Max Planck Institute of Biochemistry

ETH Zurich

University of Southern Denmark

EMD Serono

Amgen

MedImmune/AstraZeneca

MS Bioworks

Rapid Novor

Bioinformatics Solutions

Medical Proteoscope

JadeBio