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

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Trypsin is Great: But Rarely Gives the Full Picture

Protein of Interest

Trypsin Digestion & LC-MS/MS Analysis

Tryptic map
Protease 2
Protease 3

Combined Map

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)

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
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.

![Site of C-terminal Cleavage](image)

- ~56% cleavage frequency
- ~27% cleavage frequency
- ~ 6000 unique peptides
ProAlanalase Performance is Optimal around pH 1.5

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

![Unique PeptidesIdentified](chart1)
![Proline Specificity](chart2)

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\textsubscript{18}-based cleanup (e.g. C\textsubscript{18} Tips)

### Effect of Termination Method on Specificity

<table>
<thead>
<tr>
<th>Termination Method</th>
<th>Ease of Use</th>
<th>Fragmentation?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat-based</td>
<td>Easy</td>
<td>Yes: D-P bonds</td>
</tr>
<tr>
<td>C\textsubscript{18} cleanup</td>
<td>Cumbersome (multiple samples)</td>
<td>No</td>
</tr>
</tbody>
</table>

C\textsubscript{18}-Tip
Thermal

![Graph showing relative counts of different C-terminal amino acids for different termination methods.](image-url)
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

Peptide Length Distribution

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

Charge State Distribution
Effect of Denaturants on ProAlanase Activity

- 2-4M urea preferred if denaturant is needed
- *Additional denaturants may not be needed at pH 1.5!
Optimize data collection and search strategies to increase peptide IDs

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**Instrumentation**

- **Instrument**
  - Q Exactive
  - Fusion Lumos

- **Fragmentation Method**
  - HCD
  - ETD
  - HCD+ETD

**Heck Lab**

-van der Laarse et al (2019)

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**Software**

- **Search Algorithm**
  - Mascot
  - Byonic

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MS Instrument and Software Affect Results
Applications
Applications of ProAlanase

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

**New: Publication in Press**

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

[https://www.mcponline.org/content/early/2020/10/05/mcp.TIR120.002129](https://www.mcponline.org/content/early/2020/10/05/mcp.TIR120.002129)
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

<table>
<thead>
<tr>
<th></th>
<th>1:250 E:S</th>
<th>1:100 E:S</th>
<th>1:50 E:S</th>
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<tbody>
<tr>
<td></td>
<td>1 Hr</td>
<td>18 Hrs</td>
<td>1 Hr</td>
</tr>
<tr>
<td>Heavy Chain</td>
<td>97</td>
<td>79</td>
<td>98</td>
</tr>
<tr>
<td>Light Chain</td>
<td>97</td>
<td>94</td>
<td>97</td>
</tr>
</tbody>
</table>

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

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

Non-collagenous bone proteins can be used for:
- Reliable species identification
- Phylogenetic placement
- Disease identification
Paleoproteomics of the Woolly Mammoth

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

<table>
<thead>
<tr>
<th>Product</th>
<th>Amount</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>ProAlanase</td>
<td>5 µg</td>
<td>0.2 µg/µL</td>
</tr>
<tr>
<td>ProAlanase Plus</td>
<td>15 µg</td>
<td>0.5 µg/µL</td>
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**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
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Medical Proteoscope
JadeBio