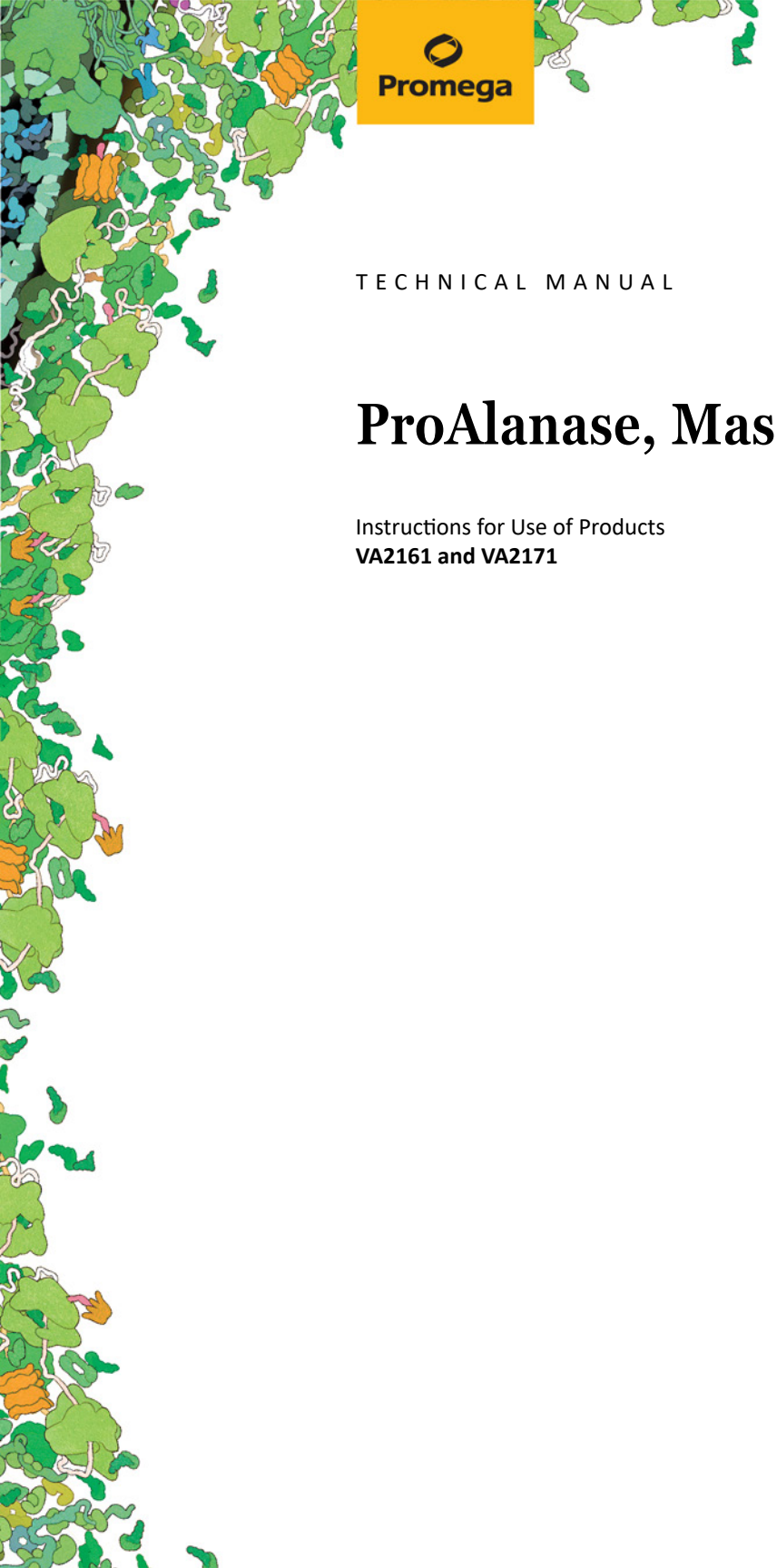

TECHNICAL MANUAL

ProAlanase, Mass Spec Grade

Instructions for Use of Products
VA2161 and VA2171

3/21
TM644

ProAlanase, Mass Spec Grade

All technical literature is available at: www.promega.com/protocols/
Visit the web site to verify that you are using the most current version of this Technical Manual.
E-mail Promega Technical Services if you have questions on use of this system: techserv@promega.com

1. Description

ProAlanase, Mass Spec Grade, is an endoprotease that preferentially cleaves proteins on the C-terminal side of proline and alanine amino acids. Isolated and purified from the fungus *Aspergillus niger*, ProAlanase is also known as An-PEP or EndoPro. Peptides derived from protein digestion with ProAlanase are suitable for identification and characterization by mass spectrometry (1–4).

Trypsin is the protease most often used in bottom-up mass spec sample preparation methods. However, using proteases other than trypsin is important. Peptides derived from other proteases can increase sequence coverage as well as identify additional proteins, post-translational modifications or both. Like trypsin, the most commonly used site-specific alternative proteases, such as Lys-C, Arg-C, Asp-N and Glu-C, cleave at charged residues. Site-specific proteases that cleave at unique sites in the proteome are needed. ProAlanase achieves this goal, preferentially cleaving proteins on the C-terminal side of proline and, to a lesser extent, alanine residues (Figure 1). Notably, ProAlanase also cleaves C-terminal to hydroxyproline residues (4).

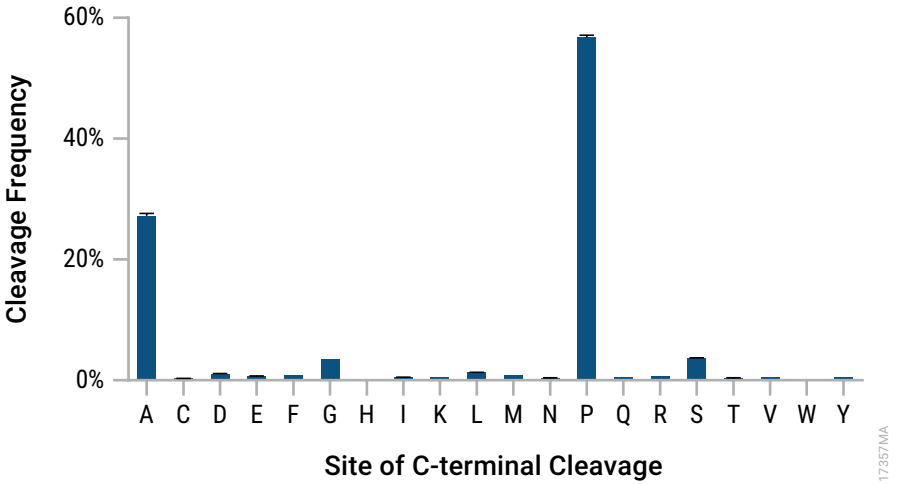

Figure 1. The C-terminal cleavage specificity of ProAlanase. MS Compatible Human Protein Extract, Intact (Cat.# V6941), was digested with ProAlanase at pH 1.5 for 2 hours at 37°C using a 1:100 enzyme:substrate ratio. Digestion was terminated by C_{18} cleanup. Peptides were analyzed by LC-MS/MS on a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. Data were searched using Byonic™ software (Protein Metrics) with no enzyme specified. Cleavage occurs predominantly on the C-terminal side of proline and alanine residues.

Perhaps more than with other proteases, ProAlanase digestion performance depends on experimental conditions. Factors such as digestion time, enzyme:substrate ratio and pH can affect digestion. Unlike most proteases used in bottom-up proteomics that cleave proteins at neutral to slightly alkaline pH, ProAlanase is active at acidic pH (~1.0–5.5). This ProAlanase feature offers several advantages. First, acidic pH of digestion can limit sample-preparation-induced artifacts such as deamidation and disulfide bond scrambling that occur under neutral and basic conditions. Second, highly acidic pH such as 1.5 can act as a chemical denaturant, facilitating the unfolding of some proteins. Finally, ProAlanase can be

used for hydrogen-deuterium exchange (HDX) mass spectrometry because acidic pH limits back-exchange of deuterium (2). Disadvantages of low-pH digestion may include the insolubility of some proteins in an acidic environment, as well as the inability to terminate digestion following commonly used protocols. While trypsin and other proteases are completely inhibited after adding strong acids, such as formic or trifluoroacetic acid (TFA), these reagents do not inhibit ProAlanase. Instead, heat ProAlanase reactions to 95°C or remove enzyme with C_{18} cleanup.

We observe optimal digestion of a complex human protein mixture with ProAlanase when the pH is 1.5-2.5 as determined both by total numbers of identified proteins and peptides (Figure 2) and digestion specificity (Figure 3). In general, digestion specificity is highest when the pH is 1.5 and digestion times are kept short; typically 2 hours or less. Longer incubation times promote nonspecific cleavage, possibly leading to undesired overdigestion (see Figure 3).

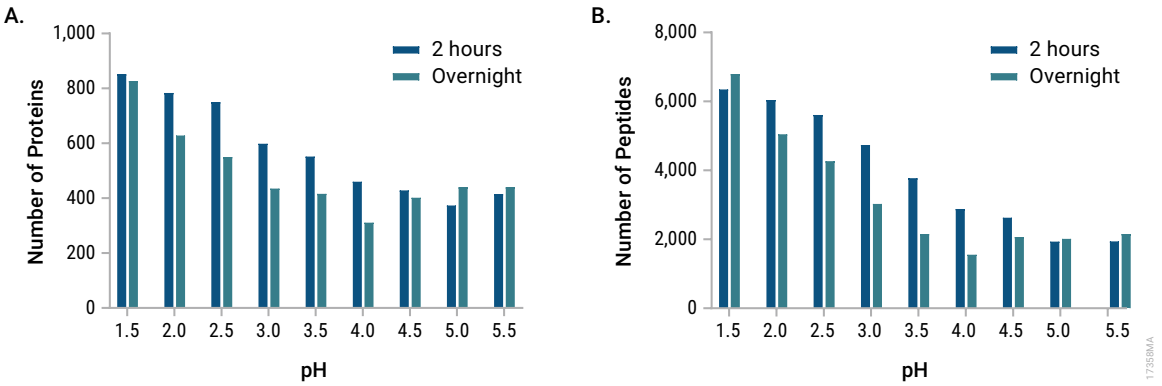

Figure 2. Protein and peptide identifications vary as a function of digestion conditions. MS Compatible Human Protein Extract, Intact (Cat.# V6941), was digested with ProAlanase at 37°C for 2 hours or overnight using a 1:50 enzyme:substrate ratio at a variety of pH values. Digestion was terminated by heating to 90°C for 10 minutes. Peptides were analyzed by LC-MS/MS on a ThermoScientific Q-Exactive Plus. Data were searched with Byonic™ software (Protein Metrics). Total numbers of identified proteins (**Panel A**) and peptides (**Panel B**) are shown.

1. Description (continued)

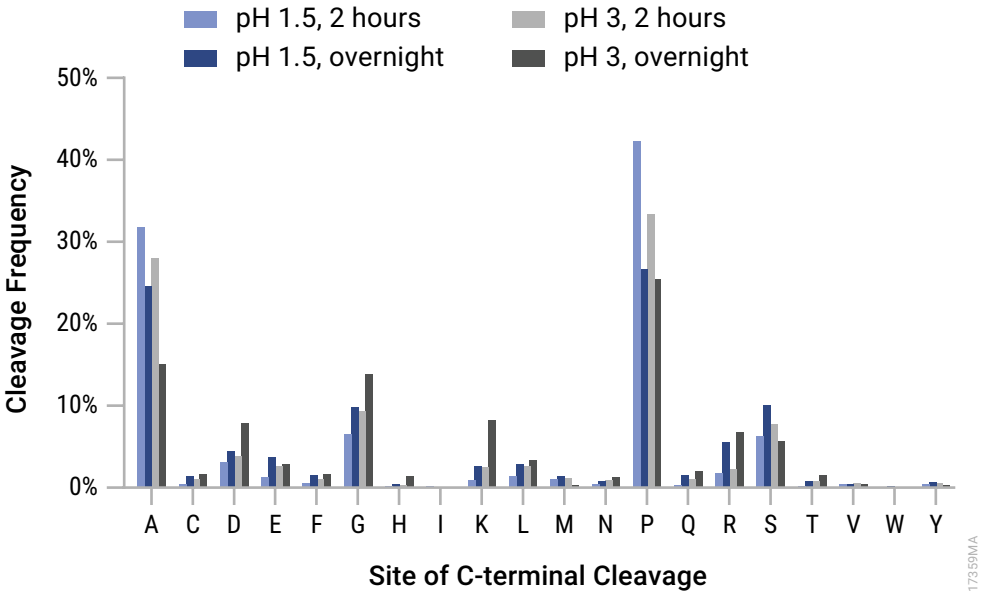

Figure 3. Digestion specificity varies as a function of digestion conditions. MS Compatible Human Protein Extract, Intact (Cat.# V6941), was digested with ProAlanase at 37°C using a 1:50 enzyme:substrate ratio at pH 1.5 or pH 3.0 for 2 hours or overnight. Digestion was terminated by heating to 90°C for 10 minutes. Peptides were analyzed by LC-MS/MS on a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer. Data were searched with Byonic™ software (Protein Metrics) with no enzyme specified. The C-terminal specificity is shown.

2. Product Components and Storage Conditions

PRODUCT	SIZE	CAT#
ProAlanase, Mass Spec Grade	**5µg**	**VA2161**
ProAlanase Plus, Mass Spec Grade	**15µg**	**VA2171**

Storage Conditions: Upon arrival, store frozen ProAlanase at –10°C or below. Prior to first use, thaw the frozen ProAlanase solution quickly in your hand and place on ice or at +2°C to +10°C. After the first thaw, ProAlanase can be stored for 2 months at +2°C to +10°C. For longer term storage, freeze and keep at –10°C or below. Do not exceed five freeze-thaw cycles.

Note: The only difference between ProAlanase, Mass Spec Grade, and ProAlanase Plus, Mass Spec Grade, is the concentration of the protease. ProAlanase, Mass Spec Grade, is provided as 0.2µg/µl solution while ProAlanase Plus, Mass Spec Grade, is a 0.5µg/µl solution.

3. Protein Preparation Prior to Digestion

To achieve efficient substrate digestion, a protease must have access to cleavage sites. Protein substrates have secondary, tertiary and sometimes quaternary structures that can impede protease access to their target cleavage sites. For that reason, protein substrates are often denatured prior to proteolytic digestion. There are numerous methods for denaturing proteins, including chemical denaturants, reducing agents, detergents, extreme pH and heat. These methods vary in their effectiveness, depending on the substrate, and often have unintended consequences such as inactivating the proteases intended for digestion. For that reason, you can minimize the denaturing effects prior to proteolytic digestion either by removing or diluting the chemical denaturants.

We recommend testing these methods when working with new proteins, or those that are particularly difficult to digest. In general, we tend to start with urea, in the range of 6–8M, followed by dilution to a range of 0.25–2M final for protein digestion. For more difficult proteins, use ~6M guanidine hydrochloride, a strong denaturant, to unfold proteins, then dilute to 0.5M or less to maintain protease activity. In the case of ProAlanase, we tested the effects of commonly used denaturants and their effect on protease activity in Figure 4. All the denaturants inhibited ProAlanase, depending on the final concentration of the chemical. The information in Figure 4 can be used as a guide when determining the concentration of denaturant to use during sample preparation.

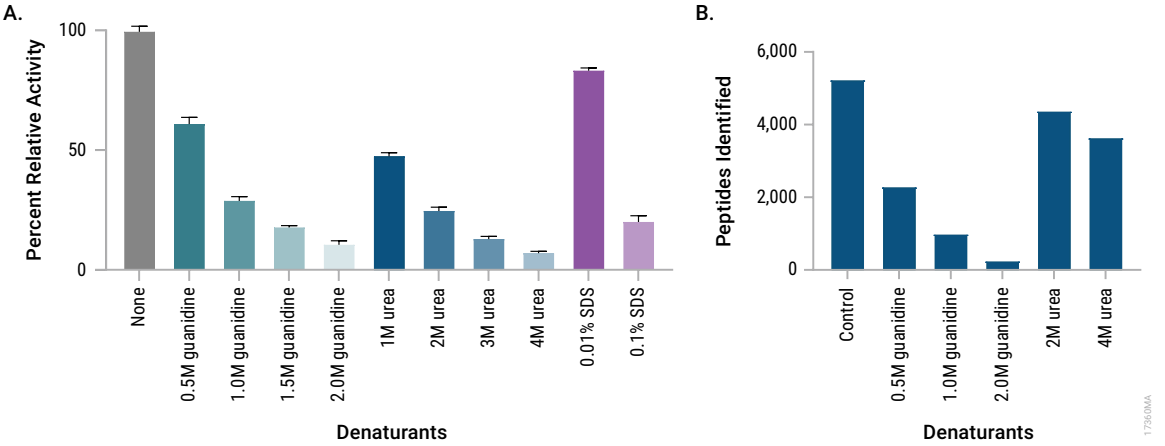

Figure 4. Effect of denaturant and concentration on enzyme activity of ProAlanase. Panel A. Synthetic peptide Z-Gly-Pro-AMC was digested at pH 4.0 in the presence or absence of various denaturants. Activity was measured by fluorescence with an excitation of 360nm and emission of 440nm. **Panel B.** MS Compatible Human Protein Extract, Intact (Cat.# V6941), was digested at 37°C for 2 hours at pH 1.5 in the presence of varying denaturants. Digests were terminated by C_{18} cleanup followed by LC-MS analysis. Total unique peptides observed after Byonic™ nonspecific search.

Paradoxically, while the intrinsic ProAlanase enzymatic activity is reduced in the presence of denaturant, in some cases, overall digestion may be enhanced due to improved access to proteolytic cleavage sites on the substrate protein(s). Furthermore, higher concentrations of enzyme can be used to overcome loss of activity induced by the denaturant. Finally, in the case of ProAlanase, the highly acidic conditions employed for digestion may also have the added benefit of denaturing certain protein substrates, eliminating the need for additional denaturants (4).

3. Protein Preparation Prior to Digestion (continued)

Materials to be Supplied by the User

- Nanopure® or equivalent grade water
- denaturant (e.g. urea or guanidine-HCl)
- digestion buffer (e.g., HCl, glycine-HCl, citrate, acetate)
- reducing agent (e.g., DTT)
- alkylating agent (e.g., iodoacetamide)

3.A. Standard Protocol for Protein Reduction and Alkylation

In this example protocol, we use the MS Compatible Human Protein Extract, Intact (Cat.# V6941), a whole-cell protein extract prepared from human K562 cells with a urea concentration of approximately 6.5M.

1. Thaw a vial of MS Compatible Human Protein Extract, Intact, (100µl at 10µg/µl).
2. Reduce by adding 1µl of 0.5M DTT (final concentration 5mM).
3. Incubate at 37°C for 30 minutes.
4. Alkylate by adding 1.5µl of 1M iodoacetamide (final concentration 15mM).
5. Incubate in the dark at room temperature for 30–60 minutes.

   Notes:

   1. Reduced and alkylated samples are ready for digestion (Section 4) or can be stored at –70°C until ready to digest.
   2. Samples may require dilution prior to digestion to reduce the urea concentration (see Section 3).

3.B. Advanced Protocol for Protein Denaturation, Reduction and Alkylation

The following is a protocol we recommend for efficient denaturing, reducing and alkylating of antibodies or other proteins that are more difficult to digest.

1. Denature the protein sample by adding guanidine HCl to a final concentration of 6M.
2. Reduce by adding DTT to a final concentration of 10mM.
3. Incubate for 30 minutes at 37°C.
4. Alkylate by adding iodoacetamide to a final concentration of 30mM.
5. Incubate in the dark at room temperature for 30–60 minutes.

   Note: At this point, the substrate is denatured, but the denaturant needs to be either diluted or removed before protein digestion. Step 6 describes one method for removing a denaturant through buffer exchange.

6. Prepare the preferred digestion buffer and perform a buffer exchange with the substrate using a desalting spin column such as a Zeba™ column. Digestion buffers for ProAlanase can include HCl or 50–100mM of buffers such as glycine, citrate or acetate. For example, for pH 1.5, add HCl to a final concentration of ~32mM. For pH 2.0, add HCl to a final concentration of 10mM.

   Notes:

   1. Check the protein concentration of your sample after desalting because yield can vary.
   2. Reduced and alkylated samples are ready for digestion (Section 4), or can be stored at –70°C until ready to digest.
   3. We have been successful when digesting antibodies with ProAlanase at pH 1.5–2 in the absence of additional denaturant, even under nonreducing conditions. The acidic pH alone may denature some proteins (4).

4. Digestion Protocol

4.A. General Considerations

ProAlanase is available in two formats that differ only in the enzyme concentration. ProAlanase, Mass Spec Grade, is provided at 0.2µg/µl while ProAlanase Plus, Mass Spec Grade, has a higher concentration of 0.5µg/µl. You can use either enzyme formulation for your experiments. There may be some instances where using a lower volume of enzyme avoids sample dilution. For those cases, we recommend using the higher enzyme concentration of ProAlanase Plus.

One of the most common considerations for digestion is the amount of enzyme to add (Figure 5). Increasing the amount of enzyme can increase digestion efficiency and may lead to a greater number of peptides detected. If specificity for proline cleavage is more important, use lower amounts of enzyme. Effects of digestion time and buffer pH are discussed in Section 1 (Figures 2 and 3).

The following guidelines can be used to help optimize your digestion protocol:

- Enzyme:substrate ratio: 1:10 to 1:500
- Digestion time: 30 minutes to overnight
- Buffer pH: 1.0 to 5.5
- Digestion buffer: HCl (pH 1–2.5), glycine HCl (pH 1–2.5), sodium citrate (pH 3–4), sodium acetate (pH 4.5–5.5)
- Denaturant concentration: Refer to Section 3 and Figure 4 for guidance.

4.A. General Considerations (continued)

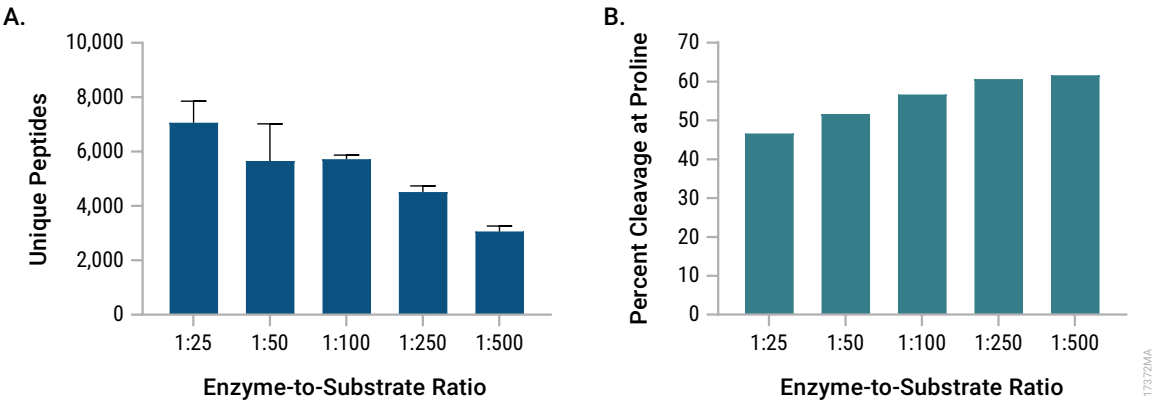

Figure 5. Effect of enzyme:substrate ratio on digestion characteristics. MS Compatible Human Protein Extract, Intact (Cat.# V6941), was digested in duplicate at 37°C for 2 hours at pH 1.5 at varying enzyme-to-substrate ratios. Digests were terminated by C_{18} cleanup followed by LC-MS analysis. **Panel A.** Total unique peptides observed after Byonic™ semispecific search. **Panel B.** Digestion specificity for proline as determined with Byonic™ nonspecific search.

4.B. Considerations for Terminating Digestion

Standard proteomics termination protocols involve inhibiting protease activity by adding formic acid or trifluoracetic acid (TFA) to acidify the sample. However, unlike trypsin and most other commonly used proteases in proteomics, ProAlanase is active at low pH.

Formic acid and TFA **will not** inhibit ProAlanase. Therefore, samples will continue to digest which may lead to overdigestion. To terminate digestions with ProAlanase, we suggest using one of the two following methods: 1) Inactivate the enzyme by heating at 90–95°C for 10 minutes, or 2) Remove the enzyme using C_{18} tips. In general, we recommend C_{18} cleanup because it yields cleaner peptide samples for downstream LC-MS. Furthermore, the cleanup method does not induce chemical cleavage between Asp-Pro bonds, which may occur under conditions where the sample is simultaneously exposed to strong acid and high temperatures.

4.C. Digesting Complex Protein Mixtures Such as Whole Cell Extracts

The following protocol serves only as a guideline. Protocols must be optimized for your substrate and desired experimental outcome.

In this example protocol, we digest 50µg of MS Compatible Human Protein Extract, Intact (Cat.# V6941), with 1µg of ProAlanase, Mass Spec Grade, (1:50 ratio) at 37°C at pH 1.5 in a final volume of 100µl. Diluting the substrate 20-fold yields a final protein concentration of 0.5µg/µl and a final urea concentration of ~0.3 M during digestion. Depending on your downstream application, you may want to avoid diluting the sample. As noted in Section 3, ProAlanase does retain some activity in the presence of denaturants (see Figure 4).

1. To digest the protein mixture, assemble the following reaction in a tube:

Reagent	Volume
reduced and alkylated human protein extract (50µg; as prepared in Section 4.B)	5µl
Milli-Q® ultrapure water	83.6µl
0.5M HCl (32mM final)	6.4µl
ProAlanase (1µg)*	5µl
Total volume	100µl

*Use 2µl of ProAlanase Plus instead. Adjust the water volume accordingly.

2. Incubate at 37°C for 2 hours.
3. Terminate the digestion mixture using one of two methods below:
   - Remove the enzyme by C_{18} cleanup (e.g. by C_{18} pipette tip). Peptides will be eluted while the enzyme remains on the tip.

   or

   - Heat to 90–95°C for 10 minutes. **Note:** High heat at acidic pH may induce chemical cleavage at peptide bonds, particularly Asp-Pro.

4.D. Digesting Difficult Proteins Such as Monoclonal Antibodies

The following protocol serves only as a guideline and protocols must be optimized for your substrate and desired experimental outcome.

In this example protocol, we digest 50µg of 1mg/ml denatured, reduced, alkylated and desalted antibody with 1µg of ProAlanase, Mass Spec Grade, (1:50 ratio) at 37°C at pH 1.5. The final volume is 100µl with a final substrate concentration of 0.5mg/ml.

1. To digest the protein mixture, assemble the following reaction in a tube:

Reagent	Volume
denatured, reduced, alkylated and desalted antibody (50µg; as prepared in Section 4.C)	50µl
Milli-Q® ultrapure water	38.6µl
0.5M HCl (32mM final)	6.4µl
ProAlanase (1µg)*	5µl
Total volume	100µl

*Use 2µl of ProAlanase Plus instead. Adjust the water volume accordingly.

2. Incubate at 37°C for 2 hours.
3. Terminate the digestion mixture using one of two methods below:
   - Remove the enzyme by C_{18} cleanup (e.g. by C_{18} tip). Peptides will be eluted while the enzyme remains on the tip.

   or

   - Heat to 90–95°C for 10 minutes. **Note:** High heat at acidic pH may induce chemical cleavage at peptide bonds, particularly Asp-Pro.

4.E. In-Gel Digestion Protocol

ProAlanase has been used successfully for in-gel digestion and we recommend using standard analysis protocols. After reduction and alkylation, in-gel protein digestion should be performed following the general considerations for ProAlanase (see Section 4.A). We recommend digesting at pH 1.5. Digestion time and enzyme amount vary, depending on how much protein is to be digested. We recommend starting with 0.2–1µg of ProAlanase and digesting for 2 hours at 37°C. Digestion can be terminated by C_{18} cleanup or heating as described in Section 4.C or 4.D.

5. Considerations for Data Collection and Data Analysis

The data collection strategy during LC-MS/MS can affect results. As reported by van der Laarse *et al.* (3), higher numbers of proteins and peptides were identified from a ProAlanase-digested HeLa extract when data were collected using electron transfer dissociation (ETD) despite fewer MS/MS scans than with higher-energy collision dissociation (HCD).

Furthermore, van der Laarse also showed that the Byonic™ software package works well when analyzing data from digests with ProAlanase (3). We observed similar results as shown in Table 1.

Table 1. Software Used May Affect Results. MS Compatible Human Protein Extract, Intact (Cat.# V6941), was digested in duplicate at 37°C for 2 hours at pH 1.5 at a 1:25 enzyme:substrate ratio. Digests were terminated by C_{18} cleanup followed by LC-MS analysis. Data were searched with a no-enzyme specification either in Mascot (Matrix Science) or Byonic (Protein Metrics). Total numbers of proteins and peptides were nearly twofold higher.

Search Software	Proteins	Unique Peptides	Percent Proline	Percent Alanine
Mascot	521	3499	39	27
Byonic	934	5952	47	31

Because there are many commercially available high-resolution mass spectrometers and software packages, we recommend trying several combinations to determine which work best for a given experiment. Customers have also reported successful searches with MaxQuant software.

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: **www.promega.com**. E-mail: **techserv@promega.com**

Symptom	Causes and Comments
Poor digestion	Ensure protein is soluble at the pH used for digestion.
	Ensure protein is efficiently unfolded so ProAlanase can access the digestion sites. Reduce and alkylate protein and add denaturants.
	Optimize digestion parameters (pH, ProAlanase amount, digestion time) to improve digestion.
	Adjust concentration of denaturant to the appropriate level for ProAlanase activity (see Figure 4).

6. Troubleshooting (continued)

Symptom	Causes and Comments
Poor digestion (continued)	ProAlanase may not be as efficient as an enzyme like trypsin. Typical digestion efficiency at proline residues is around 60% although this efficiency can be achieved quickly, typically in 2 hours or less. Missed cleavages at proline tend to occur when basic amino acids are located two amino acids to the C-terminal side of the proline residue. Missed cleavage at proline may also occur when the cleavage site is flanked by an adjacent glycine on the N-terminal side or an additional adjacent proline on the C-terminal side. ProAlanase is less efficient for digestion at alanine residues than it is at proline residues. The mechanism for this observation is not understood, but ProAlanase appears to have some general selectivity for small amino acids, which is why cleavage at serine and glycine is also observed.
Low specificity	Reduce the amount of enzyme used.
	Reduce the digestion time.
	Digestion was not effectively terminated, leading to continued digestion in the autosampler. Unlike most proteases, the ProAlanase reaction cannot simply be stopped by adding TFA or formic acid. We recommend two methods to efficiently stop digestion: 1) Remove the enzyme with C_{18} cleanup which also cleans up peptides prior to LC-MS analysis. 2) Heat digest reaction at 95°C for 10 minutes. Boiling samples at low pH may introduce artificial cleavage events particularly between Asp-Pro bonds.
	ProAlanase has some general specificity for small amino acids. Therefore, cleavage at serine and glycine may also be observed.
Few proteins identified	Identification rates from proteases other than trypsin tend to be lower.
	Consider changing data collection methods. van der Laarse *et al.* (3) suggest that ETD and electron transfer higher-energy collision dissociation (EThcD) outperform HCD/collision-induced dissociation (CID).
	Consider using the most advanced mass spectrometer available. For example, we have observed higher protein and peptide identifications on tribrid orbitrap instruments than on quadrupole orbitrap instruments.

Symptom	Causes and Comments
Few proteins identified (continued)	Optimize the bioinformatics approach. In our experience, Byonic™ software provides a higher number of protein and peptide IDs.
	Some protein samples may precipitate at low pH. Consider using additives to increase protein solubility or alternate digestion methods that work with aggregated or suspended proteins such as SP3 (5), PAC (6) or S-Trap™ sample processing.

7. References

1. Sebela, M. *et al.* (2009) Identification of N-glycosylation in prolyl endoprotease from *Aspergillus niger* and evaluation of the enzyme for its possible application in proteomics. *J. Mass. Spectrom.* **44**, 1587–95.
2. Tsiatsiani, L. *et al.* (2017) *Aspergillus niger* prolyl endoprotease for hydrogen-deuterium exchange mass spectrometry and protein structural studies. *Anal. Chem.* **89**, 7966–73.
3. van der Laarse, S.A.M. *et al.* (2020) Targeting proline in (phospho)proteomics. *FEBS J.* **287**, 2979–97.
4. Samodova, D. *et al.* (2020) ProAlanase is an effective alternative to trypsin for proteomics applications and disulfide bond mapping. *Mol. Cell. Proteomics* **19**, 2139–57.
5. Hughes, C.S. *et al.* (2014) Ultrasensitive proteome analysis using paramagnetic bead technology. *Mol. Syst. Biol.* **10**, 757.
6. Batth, T.S. *et al.* (2019) Protein aggregation capture on microparticles enables multipurpose sample preparation. *Mol. Cell. Proteomics* **18**, 1027–35.

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8. Related Products

Product	Size	Cat.#
Arg-C, Sequencing Grade	10µg	V1881
Chymotrypsin, Sequencing Grade	25µg	V1061
	100µg (4 × 25µg)	V1062
Glu-C, Sequencing Grade	50µg (5 × 10µg)	V1651
MS Compatible Human Protein Extract, Intact	1mg	V6941
Pepsin	250mg	V1959
PNGase F	500u 10u/µl	V4831
rAsp-N, Mass Spec Grade	10µg	VA1160
rLys-C, Mass Spec Grade	15µg	V1671
Sequencing Grade Modified Trypsin	100µg (5 × 20µg)	V5111
	100µg	V5117
Sequencing Grade Modified Trypsin, Frozen	100µg (5 × 20µg)	V5113
Trypsin Gold, Mass Spectrometry Grade	100µg	V5280
Trypsin/Lys-C Mix, Mass Spec Grade	20µg	V5071
	100µg	V5072
	100µg (5 × 20µg)	V5073
IdeS Protease	5,000 units	V7511
	25,000 units (5 × 5,000 units)	V7515
IdeZ Protease	5,000 units	V8341
	25,000 units (5 × 5,000 units)	V8345