

Using Endoproteinases Asp-N and Glu-C to Improve Protein Characterization

ABSTRACT

Two alternative proteinases, Asp-N Sequencing Grade and Glu-C Sequencing Grade are available from Promega to expand options for protein digestion. Asp-N, Sequencing Grade, is a metallo proteinases that preferentially cleaves at the N-terminus of aspartic and cysteic acid residues. Glu-C is a serine proteinases that cleaves with high specificity at the C-terminus of glutamic and aspartic acids. Use of alternative enzymes for protein digestion increases the confidence in mass spectrometry data by confirming the protein sequence and aids in the mapping of post-translational modifications (PTMs). Here we describe the cleavage specificity of endoproteinases Asp-N and Glu-C, and show an example of improved protein sequence coverage through the use of alternative digestion with these enzymes.

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Introduction

Endoproteinases Asp-N and Glu-C have been used for protein characterization for over 30 years and have gained importance recently due to advancements in mass spectrometry techniques. Asp-N preferentially cleaves proteins at the N-terminus of aspartic and cysteic acid^{(1) (2) (3)}. Glu-C cleaves at the C-terminus of glutamic and aspartic residues^{(4) (5) (6)}. Due to their specific cleavage sites, these proteinases create unique peptide fragments available for mass spectrometry analysis. Comparing protein sequences or mapping data from Asp-N or Glu-C digests to that of other proteinases promotes higher confidence in data^{(7) (8) (9) (10) (11) (12)}.

Protein digestion is required for either the bottom-up or middle-down approach to protein analysis. In the bottom-up approach, the optimal peptide size range for analysis is 600–5,000Da. Recent advancements in mass spectrometry such as electron transfer dissociation (ETD) and electron capture dissociation (ECD), the potential to combine with collision activated dissociation (CAD) and in software, have expanded the peptide size range to 600–20,000Da. These advancements have promoted the middle-down approach to protein analysis^{(13) (14) (15) (16) (9)}. However, some peptides still may fall above or below the the desired peptide size range. This results in decreased protein coverage and incomplete data collection.

To increase protein coverage, additional proteinases have been used. Using an alternative proteinases individually or in combination with other proteinases creates a unique peptide map that may include sequences not seen in trypsin digestions. Overlaying peptides obtained from digestion with alternative proteinases increases protein coverage and overall confidence in protein identification. When alternative enzymes are used, larger peptides are cleaved into smaller fragments, which are more manageable for the instrumentation. Conversely, protein sections cleaved into peptides too small for analysis by one enzyme might be cleaved into larger peptides with a different enzyme. Alternative proteinases also help to overcome incomplete digestion caused by PTMs, which prevent the proteinases from accessing that particular site. By using alternative enzymes, the protein might be cleaved at sites further away from the PTM. The examples above show that alternative proteinases are beneficial to protein analysis^{(13) (14) (15) (16) (9)}.

Asp-N, Sequencing Grade

Asp-N is a metalloprotease purified from *Pseudomonas fragi*. Asp-N cleaves at the N-terminus of aspartic and cysteic acid residues with high specificity(1) (2) (3) . Cysteic acid residues are an oxidized form of the cysteine residues. Additional cleavage has been reported at glutamic residues; however, the rate of cleavage at aspartic acid is 2,000-fold higher than at the glutamyl residues(2) (3) . To demonstrate cleavage specificity, we over digested glucagon using a 1:10 Asp-N to peptide ratio (w/w). Samples from 1 and 18 hours of digestion were analyzed using C18 reverse-phase chromatography and compared. Digestion of glucagon with Asp-N creates four peptides. A change in the cleavage pattern would indicate nonspecific cleavage by Asp-N. The results showed the four peaks were unchanged after 18 hours of digestion, which indicated high cleavage specificity (Figure 1).

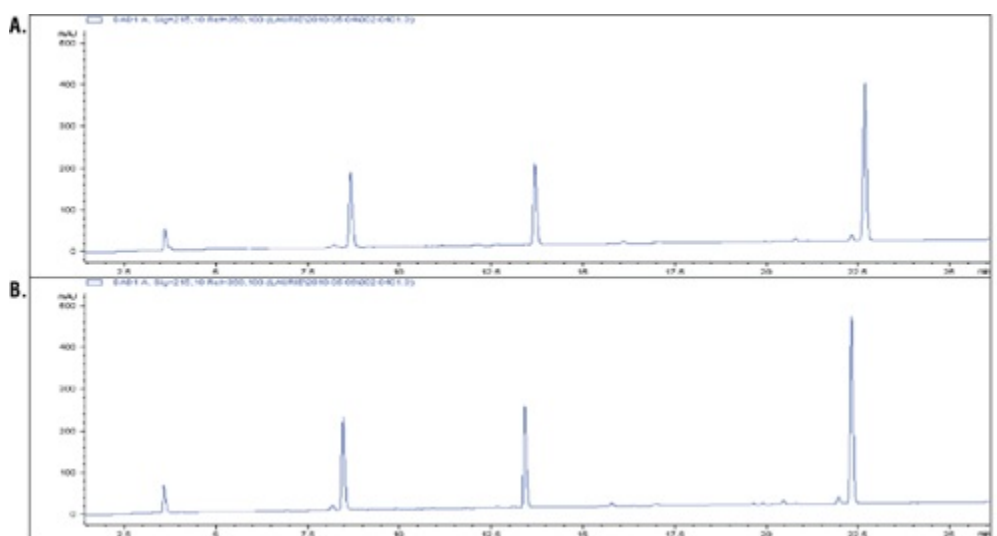


Figure 1. Overdigestion of glucagon with endoproteinase Asp-N. Glucagon (20µg) was digested with 2µg of Asp-N in ammonium bicarbonate buffer (pH 7.5) at 37°C. Following 1 and 18 hours of digestion, the samples were analyzed on an Agilent Technologies 1200 Series HPLC instrument. Peptides were separated using a Zorbax SB-C18 3.5µm, 3.0 × 100mm column solvent A, 0.1% TFA; Solvent B, 0.1% TFA in acetonitrile. Gradient, 2.5–55% B; flow rate of 0.75ml/minute; detected at 215nm. **Panel A.** One-hour digestion. **Panel B.** Eighteen-hour digestion.

Glu-C, Sequencing Grade

Glu-C is a serine proteinase purified from *Staphylococcus aureus* V8. It cleaves specifically at the C-terminus of glutamic and aspartic acids(4) (5) (6) . Buffer composition effects the specificity of Glu-C. In phosphate buffers, both glutamic and aspartic residues are cleaved; however, in ammonium bicarbonate and ammonium acetate buffers (pH 4.0), only the glutamic residues are cleaved(6) . The specificity of Glu-C was demonstrated by overdigesting insulin β-chain in a 1:10 Glu-C-to-protein ratio (w/w) in ammonium bicarbonate buffer. The digestion of insulin β-chain by Glu-C creates three peaks in the C18 reverse-phased analysis. Additional peaks in the chromatographs would indicate nonspecific cleavage activity. Figure 2 shows that the peak patterns apparent after 1 hour of digestion (Panel A) remain unchanged after 18 hours of digestion, indicating cleavage specificity (Panel B).

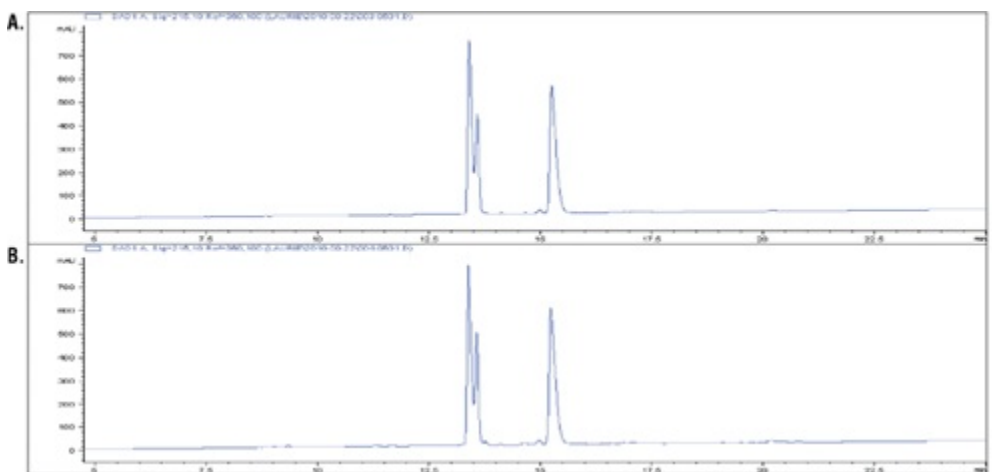


Figure 2. Overdigestion of insulin β -chain by Glu-C. One hundred micrograms of insulin β -chain was digested with 10 μ g of Glu-C in ammonium bicarbonate buffer (pH 7.5) at 37°C. Following 1 and 18 hours of digestion, the samples were analyzed on an Agilent Technologies 1200 Series HPLC instrument. Peptides were separated using a Zorbax SB-C18 3.5 μ m, 3.0 \times 100mm column, solvent A, 0.1% TFA; Solvent B, 0.1% TFA in acetonitrile. Gradient, 2.5–55% B flow rate of 0.75ml/minute, detected at 215nm. **Panel A.** One-hour digestion. **Panel B.** Eighteen-hour digestion.

Multiple Enzyme Digestion

Digesting a protein with alternative enzymes, either individually or in combination, is useful for increasing the sequence coverage of a protein, identifying PTM sites and identifying translocation partners. To demonstrate the use of alternative proteinases for increasing protein sequence coverage, we digested β -casein with either Asp-N, Glu-C or trypsin for 18 hours. The reactions were stopped and analyzed using liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS). The results of the digestions were as follows: Glu-C, 49%; Asp-N, 37%; and trypsin, 49%. After overlaying the peptide maps obtained from each digestion, the total coverage increased to 73% (Figure 3).

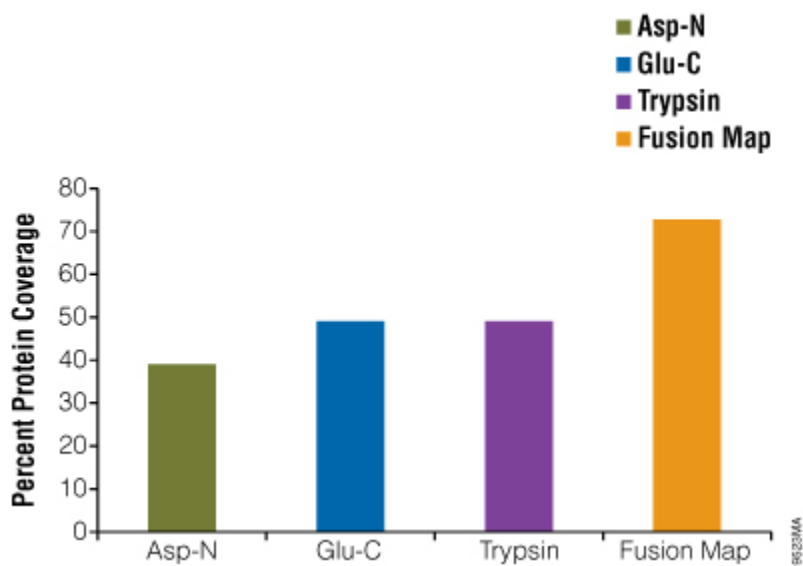


Figure 3. Increasing protein sequence coverage using multiple enzymes. β -Casein was digested with Glu-C, Asp-N or trypsin overnight. Samples were analyzed using LC/MS/MS on a LTQ Orbitrap XL. The results were searched against the forward and reverse Uniprot Bovine database using the Mascot search engine to determine the protein sequence coverage. Individual sequence coverage for the digestions were: Glu-C, 49%; Asp-N, 39%; trypsin, 49%. When the individual peptide maps were overlaid, the protein coverage increased to 73%.

Conclusion

We show here that Asp-N and Glu-C, Sequencing Grade, endoproteases are excellent tools that can be used to improve protein analysis. Both proteinases demonstrate high cleavage specificity. Asp-N, Sequencing Grade, proteinase specifically cleaves at the N-terminus of the aspartic and cysteic acid residues. Glu-C, Sequencing Grade, proteinase cleaves at the C-terminus of glutamic and aspartic residues. These enzymes create different peptides compared to those created by trypsin digestion, offering higher protein coverage and an overall increase in protein identification. In the model experiment with β -casein, we showed that incorporation of Asp-N and Glu-C into the digestion protocol led to a 24% increase in protein coverage when compared with the coverage with trypsin alone. Asp-N and Glu-C, Sequencing Grade, endoproteases also offer an efficient way to protein characterization. protein sequence coverage and PTM mapping.

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