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Product Information

Protease Profiler™ Kit

Catalog Number **PP0500**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Proteolytic digestion of an unknown sample protein is often used to determine or confirm the identity of the protein of interest. Use of proteolytic enzymes with specific cleavage sites results is a limited number of peptide products, which may be further analyzed to characterize the unknown protein.

A double enzymatic digestion may be necessary to produce peptide fragments for analysis. The initial digestion may result in long peptide fragments (>5,000 Da) that are often not detected by mass spectrometry (MS). A second digestion using a protease with a different specificity results in cleavage of the larger peptides, producing small peptides suitable to MS analysis. For example, trypsin, Asp-N, and Glu-C can be used for double digests as they have different specificities.[1,2]

The Protease Profiler Kit contains 5 proteolytic enzymes, an Enzyme Solubilization Reagent, and an Enzyme Reaction Buffer. It allows customers to try different enzymatic digestions on their proteins of interest. In-solution digestion procedures are provided for the proteolytic enzymes. An in-gel procedure is also provided (see Appendix).

Components

The Protease Profiler Kit contains the following seven components:

- Asp-N (Catalog Number P3303) 2 µg
- Glu-C (Catalog Number P6181) 50 µg
- Lys-C (Catalog Number P3428) 5 µg
- Arg-C (Catalog Number P6056) 5 µg
- Trypsin (Catalog Number T6567) 20 µg
- Trypsin Solubilization Reagent (Catalog Number T2073) – Reagent for reconstitution and stabilization trypsin and Arg-C enzymes. 1 ml
- Enzyme Reaction Buffer (Catalog Number E0530) 25 ml

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The entire Protease Profiler Kit retains activity for at least 1 year when stored at –20 °C. Alternatively, some of the components (Trypsin, Lys-C, Asp-N, Trypsin Solubilization Reagent, and Enzyme Reaction Buffer) may be stored at 2–8 °C. The storage temperature indicated on the labels of these products is 2–8 °C. Storage at –20 °C will not compromise the performance of these products.

Preparation Instructions

Trypsin Solubilization Reagent (Catalog Number T2073) - The reagent is a 1 mM HCl solution and is ready to use. No change in the performance of the solution is observed for 1 year when stored at 2–8 °C or –20 °C.

Enzyme Reaction Buffer (Catalog Number E0530) – The Enzyme Reaction Buffer is prepared by adding 25 ml of water to the contents of the bottle. This results in a solution of 100 mM ammonium bicarbonate with a pH of ~8.0. The reconstituted Enzyme Reaction Buffer may be stored for up to 2 weeks at 2–8 °C or may be stored at –20 °C for up to one year. Freeze-thaw cycles do not affect the performance of this buffer.

Sample Protein

For an in-solution digestion, reconstitute a solid or lyophilized purified protein preparation in the Enzyme Reaction Buffer. A protein preparation in solution should be dialyzed overnight against 100 mM ammonium bicarbonate. This is especially important for protein solutions with large volumes at extreme pH. Such solution samples may shift the pH of the digestion buffer resulting in less enzyme activity.

High concentrations (>5 mM) of Tris-HCl buffer in samples are incompatible with MALDI-MS analysis and the Enzyme Reaction Buffer should be used when using MALDI-MS analysis. A sample protein concentration of 1 mg/ml is typically used, but other concentrations are also acceptable.

Note: Effective digestion of the sample protein may require relaxation of the secondary structure or partial denaturation of the sample protein prior to proteolytic digestion. Several methods may be used to increase the effectiveness of a digestion. These methods may be combined to increase effectiveness of digestion.

1. It is recommended to reduce and alkylate the sample protein prior to enzyme digestion. Reduction and alkylation disrupt the disulfide bonds relaxing the secondary protein structure, making the cleavage sites more accessible. The ProteoPrep® Reduction and Alkylation Kit (Catalog Number PROTRA) contains reagents and procedures for reduction and alkylation of the sample protein.
2. Heat denaturation of the sample protein may result in a more effective enzymatic digestion.[50] It is suggested to heat the sample protein (not the proteolytic enzyme) for 10 minutes at 100 °C prior to the digestion. After heating, transfer the sample protein immediately to ice, cool, then add the enzyme, and continue with digestion.
3. For sample proteins having dense secondary structure, the use of an organic solvent may result in a more effective enzymatic digestion. Digestions may be performed with 30% acetonitrile in the reaction buffer for trypsin, Lys-C, and Asp-N. The acetonitrile relaxes the secondary structure of the sample protein and allows the proteolytic enzyme better access to the cleavage sites. The addition of acetonitrile to the above enzymatic digestions greatly enhances the rate of the digestion. With 30% acetonitrile in the reaction buffer, a 30 minute trypsin reaction resulted in ~95% digestion of reduced and alkylated carbonic anhydrase II.

Procedures for In-solution Digestions

Asp-N (Catalog Number P3303)
EC 3.4.24.33
CAS RN 9001-92-7

Endoproteinase Asp-N is a metallo-endoprotease, isolated from a mutant strain of *Pseudomonas fragi*, which hydrolyzes peptide bonds on the N-terminal side of aspartic and cysteic acid residues.[3-5] It has an average molecular mass of 24.5 kDa and a pH optimum between 6.0–8.5.[3]

This product is HPLC purified and is suitable for proteomic work. In either reaction buffer, the Enzyme Reaction Buffer or 100 mM Tris-HCl, pH 8.5, Asp-N specifically cleaves peptide bonds on the N-terminal side of aspartic and cysteic acid residues.[3-5]

Asp-N is used in proteomics for peptide mapping and protein sequence work due to its highly specific cleavage of peptides resulting in a limited number of peptide fragments.[3-8]

The product is supplied in a vial containing 2 µg of lyophilized Asp-N with Tris-HCl. The lyophilized product is reconstituted with 50 µl of water, resulting in a protease solution containing 10 mM Tris-HCl, pH 8.0. The lyophilized powder retains activity for at least one year if stored desiccated at 2–8 °C. After reconstitution with water, frozen aliquots can be stored for several weeks.[3,5]

Solution Digestion Procedure

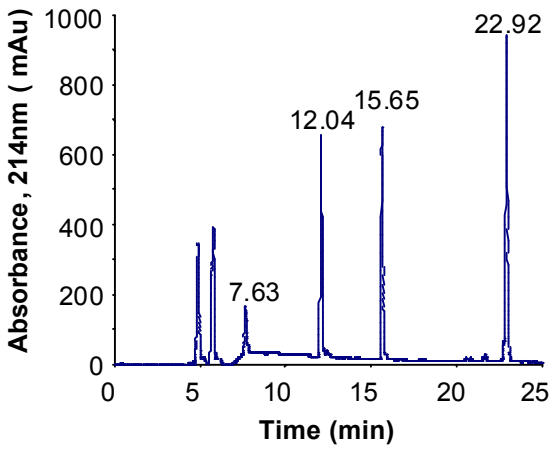
Self-digestion may occur if digestion temperatures above 37 °C are used. Asp-N retains most of its activity in 2.0 M urea, 1.0 M guanidine HCl, or 0.1% SDS.[3,8] A known peptide such as glucagon should be run as a control for all experiments.

1. Add the prepared Asp-N enzyme solution to the sample protein solution. A ratio between 1:50 and 1:200 (w/w) of Asp-N enzyme to protein substrate is recommended.
2. Incubate the reaction at 37 °C for 2 hours to overnight depending on the enzyme to substrate ratio.
3. Digest is ready to be analyzed by HPLC-MS or MALDI-MS (see Figure 1).

Figure 1.
Asp-N digestion of glucagon (Catalog Number G7774)

The sequence of glucagon is:

HSQGTFTSDYSKYLDSRRAQDFVQWLMNT



Glucagon (40 µg) was digested with 2 µg of Asp-N for 18 hours at 37 °C in Enzyme Reaction Buffer. A 20 µg aliquot was separated on a Supelco Discovery® C18 column (25 cm × 4.6 mm, 5 micron, Catalog Number 504971) using a 20 minute linear gradient from 5–50% B at 0.7 ml/min with UV detection at 214 nm followed by mass spectrometry.
Solvent A: 0.1% (v/v) TFA in water.
Solvent B: 0.08% (v/v) TFA in acetonitrile.

The Asp-N proteolytic fragments were identified as follows:

Retention Time (min)	Mass (Da)	Fragment
7.63	731.3	Asp^{15}-Gln^{20}
12.04	863.3	His^{1}-Ser^{8}
15.65	787.3	Asp^{9}-Leu^{14}
22.92	1152.3	Asp^{21}-Thr^{29}

During the 18 hour digestion only the expected peptide fragments were generated with no indication of other major proteolytic activity.

Lys-C (Catalog Number P3428)
EC 3.4.21.50
CAS RN 72561-05-8

Endoproteinase Lys-C from *Lysobacter enzymogenes* is a serine endoprotease, which hydrolyzes peptide bonds on the carboxyl side of lysyl residues.[8-13] Lys-Pro and Lys-Glu bonds are also cleaved.[12] Some minor non-specific cleavage has been reported.[8,12] The protease readily cleaves at aminoethylcysteine residues.[13] It has an average molecular mass of 27.96 kDa and a pH optimum of ~8.5.[9]

This product is HPLC purified and is suitable for proteomic work. In either reaction buffer, the Enzyme Reaction Buffer or 100 mM Tris-HCl, pH 8.5, Lys-C specifically cleaves peptide bonds on the carboxyl side of lysine. It is widely used in proteomics for peptide mapping and protein sequence work due to this highly specific cleavage of peptides resulting in a limited number of fragments.[8-13]

The product is supplied in a vial containing 5 µg of lyophilized Lys-C with Tricine and EDTA. The lyophilized product is reconstituted with 50 µl of water, resulting in a protease solution containing 50 mM Tricine, pH 8.0, with 10 mM EDTA. The lyophilized powder retains activity for at least one year if stored desiccated at 2–8 °C. The hygroscopic nature of the lyophilized powder may make it appear wet. The stability and suitability of the enzyme is not affected. After reconstitution in water, frozen aliquots can be stored for several weeks.[11]

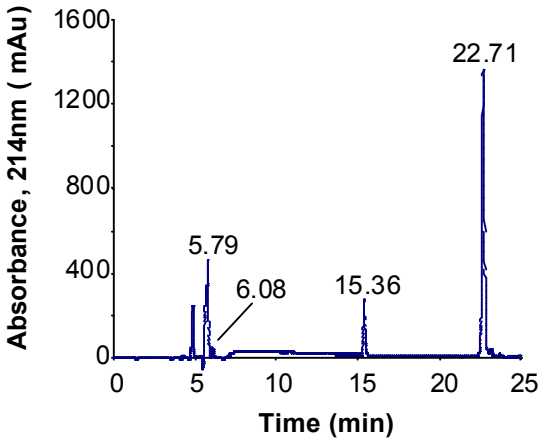
Solution Digestion Procedure
Self-digestion may occur if digestion temperatures above 37 °C are used. Lys-C retains most of its activity in 2.0 M urea or 0.1% SDS.[8,10,13] A known peptide such as melittin or the oxidized B chain of insulin should be run as a control for all experiments.

1. Add the prepared Lys-C enzyme solution to the sample protein solution. A ratio between 1:20 and 1:100 (w/w) of Lys-C enzyme to protein substrate is recommended.
2. Incubate the reaction at 37 °C for 2 hours to overnight depending on the enzyme to substrate ratio.
3. Digest is ready to be analyzed by HPLC-MS or MALDI-MS (see Figure 2).

Figure 2.
Lys-C digestion of melittin (Catalog Number M4171)

The sequence of melittin is:

GIGAVLKVLTTGLPALISWIKRKRQQ



Melittin (100 µg) was digested with 5 µg of Lys-C for 18 hours at 37 °C in 100 µl of Enzyme Reaction Buffer. A 20 µg aliquot was separated on a Supelco Discovery C18 column (25 cm × 4.6 mm, 5 micron, Catalog Number 504971) using a 20 minute linear gradient from 5–50% B at 0.7 ml/min with UV detection at 214 nm followed by mass spectrometry.
Solvent A: 0.1% (v/v) TFA in water.
Solvent B: 0.08% (v/v) TFA in acetonitrile.

The Lys-C peptide fragments were identified as follows:

Retention Time (min)	Mass (Da)	Fragment
5.79	429.3	Arg^{24}-Gln^{26}
6.08	302.3	Arg^{22}-Lys^{23}
15.36	656.4	Gly^{1}-Lys^{7}
22.71	1512.0	Val^{8}-Lys^{21}

The two short hydrophilic peptides (retention times of 5.79 and 6.08) co-elute with the unbound buffer salts in the injection peak. The retention times for these two peptides were determined by searching for their expected masses.

During the 18 hour digestion only the expected peptide fragments were generated with no indication of other major proteolytic activity. Under the experimental conditions the cleavage of the test peptide was complete in less than one hour.

Glu-C (Catalog Number P6181)
EC 3.4.21.19
CAS RN 66676-43-5

Endoproteinase Glu-C from *Staphylococcus aureus* strain V8 is a serine endoprotease, which hydrolyzes peptide bonds on the carboxyl side of glutamyl and aspartyl residues. The specificity of Glu-C is dependent upon the buffer and pH employed as well as the structure around the potential cleavage site.[8-11,14-16] In the Enzyme Reaction Buffer or ammonium acetate buffer, pH 4.0, the enzyme preferentially cleaves glutamyl bonds; whereas, in phosphate buffer, pH 7.8, Glu-C will cleave at either site. No cleavage will occur if a proline residue is on the carboxyl side.[14] The enzyme also exhibits esterase activity.[14,15] It has an average molecular mass of 29.02 kDa.[17,18] The protease is active in the pH range of 3.5–9.5 and exhibits a double maximum of activity at pH 4.0 and 7.8 with hemoglobin as the substrate.[14,15]

This product is suitable for proteomic work. Glu-C is widely used in proteomics for peptide mapping and protein sequence work due to its highly specific cleavage of peptides resulting in a limited number of fragments.[8-11,14-16]

The product is supplied in a vial containing 25 µg of lyophilized Glu-C. The lyophilized product is reconstituted with 250 µl water. The lyophilized preparation, which may appear as a clear film, retains activity for at least one year if stored at –20 °C. The frozen solution can be thawed and frozen repeatedly without loss of activity.[14]

Solution Digestion Procedure
The specificity and activity of Glu-C is retained in 20% organic solvents.[26] Glu-C is reported to be fully active in the presence of 0.2% SDS and retains 50% of its activity in 4.0 M urea.[9-11,15,16] A known peptide such as the oxidized B chain of insulin should be run as a control for all experiments.

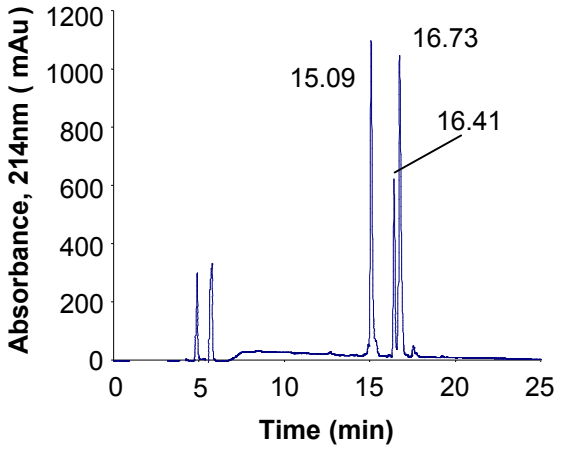
1. Add the prepared Glu-C enzyme solution to the sample protein solution. A ratio between 1:20 and 1:100 (w/w) of Glu-C enzyme to protein substrate is recommended.
   Note: To increase the digestion rate of Glu-C, the enzyme:substrate ratio can be changed to 1:1 and the digestion extended to 40 hours. This may be helpful with sample proteins that are difficult to cleave.[1]
2. Incubate the reaction at 37 °C for 2 hours to overnight depending on the enzyme to substrate ratio.

3. Digest is ready to be analyzed by HPLC-MS or MALDI-MS (see Figure 3).

Figure 3.
Glu-C digestion of the oxidized B chain of insulin (Catalog Number I1764)

The sequence of the oxidized B chain of insulin is:

FVNQHLC$_{ox}$GSHLVEALYLVC$_{ox}$GERGFFYTPKA



The oxidized B chain of insulin (100 µg) was digested with 5 µg of Glu-C for 18 hours at 37 °C in 100 µl of Enzyme Reaction Buffer. A 20 µg aliquot was separated on a Supelco Discovery C18 column (25 cm × 4.6 mm, 5 micron, Catalog Number 504971) using a 20 minute linear gradient from 5–50% B at 0.7 ml/min with UV detection at 214 nm followed by mass spectrometry.
Solvent A: 0.1% (v/v) TFA in water.
Solvent B: 0.08% (v/v) TFA in acetonitrile.

The Glu-C peptide fragments were identified as follows:

Retention Time (min)	Mass (Da)	Fragment
15.09	1529.4	Phe^{1}-Glu^{13}
16.41	914.3	Ala^{14}-Glu^{21}
16.73	1086.0	Arg^{22}-Ala^{30}

During the 18 hour digestion only the expected peptide fragments were generated with no indication of other major proteolytic activity.

Arg-C (Catalog Number P6056)
EC 3.4.21.40
CAS RN 82047-85-6

Endoproteinase Arg-C is a serine endoprotease from the mouse submaxillary gland, which hydrolyzes peptide bonds on the carboxyl side of arginyl residues and also exhibits esterase and amidase activities.[19,20] The enzyme has been shown to cleave Lys-Lys and Lys-Arg bonds, and all Arg-X bonds may not be hydrolyzed.[8,10,21-22] It has an average molecular mass of 26.5 kDa and a pH optimum between 7.5–8.5.[19,20]

This product is HPLC purified and is suitable for proteomic work. In either reaction buffer, the Enzyme Reaction Buffer or 100 mM Tris-HCl, pH 8.5, Arg-C specifically cleaves peptide bonds on the carboxyl side of arginine. Arg-C is used in proteomics for peptide mapping and protein sequence work due to its highly specific cleavage of peptides resulting in a limited number of fragments.[8,10,19-22]

The product is supplied in a vial containing 5 µg of lyophilized Arg-C. The lyophilized product is reconstituted with the Trypsin Solubilization Reagent (1 mM HCl). The lyophilized powder retains activity for at least one year if stored at –20 °C. After reconstitution in 1 mM HCl, frozen aliquots lose 50% activity after one week.
Note: Alternately, Arg-C may be reconstituted with a solution of 50 mM ammonium bicarbonate, pH 8.0, with 1 mM calcium acetate and 7.5 mM DTT. Another suggestion is to raise the pH of the digestion reaction buffer to 8.5 by adding ammonium hydroxide.

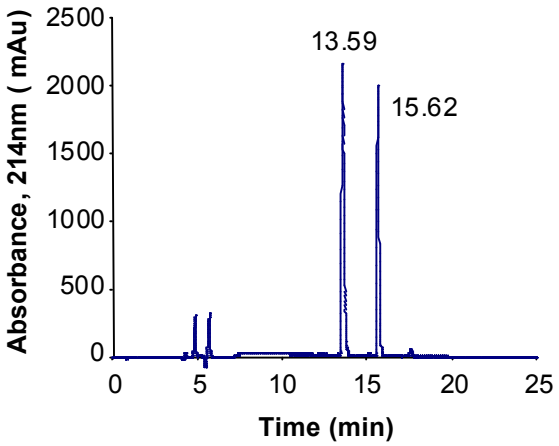
Solution Digestion Procedure
Self digestion may occur during the alkaline incubation period. The specificity and activity of Arg-C is retained in 20% organic solvents.[26] Endoproteinase Arg-C loses specificity during long incubations. A known peptide such as α-melanocyte stimulating hormone should be run as a control for all experiments.

1. Add the prepared Arg-C enzyme solution to the sample protein solution. A ratio between 1:20 and 1:100 (w/w) of Arg-C enzyme to protein substrate is recommended.
2. Incubate the reaction at 37 °C for 2 hours to overnight depending on the enzyme to substrate ratio.
3. Digest is ready to be analyzed by HPLC-MS or MALDI-MS (see Figure 4).

Figure 4.
Arg-C digestion of α-melanocyte stimulating hormone
Catalog Number M4135)

The sequence of α-melanocyte stimulating hormone is:

Acetyl-SYSMEHFRWGKPV



α-Melanocyte stimulating hormone (100 μg) was digested with 5 μg of Arg-C for 18 hours at 37 °C in 100 μl of Enzyme Reaction Buffer. A 20 μg aliquot was separated on a Supelco Discovery C18 column (25 cm × 4.6 mm, 5 micron, Catalog Number 504971) using a 20 minute linear gradient from 5–50% B at 0.7 ml/min with UV detection at 214 nm followed by mass spectrometry.
Solvent A: 0.1% (v/v) TFA in water.
Solvent B: 0.08% (v/v) TFA in acetonitrile.

The Arg-C proteolytic fragments were identified as follows:

Retention Time (min)	Mass (Da)	Fragment
13.59	584.3	Trp^{9}-Val^{13}
15.62	1098.0	Acetyl-Ser^{1}-Arg^{8}

During the 18 hour digestion only the expected peptide fragments were generated with no indication of other major proteolytic activity.

Trypsin (Catalog Number T6567)
EC 3.4.21.4
CAS RN 9002-07-7

Trypsin is routinely used in proteomics for peptide mapping and protein sequence work, due to its highly specific cleavage resulting in a limited number of peptide fragments.[8-11,23] Trypsin is a pancreatic serine endoprotease, which hydrolyzes peptide bonds specifically on the carboxyl side of arginine and lysine residues. The rate of hydrolysis is slower if an acidic residue is on either side of the cleavage site and cleavage may not occur if a proline residue is on the carboxyl side.[8-11,23] The enzyme also exhibits esterase and amidase activities.[23] Trypsin has an average molecular mass of 23.29 kDa and a pH optimum near 8.0.[23]

This product has been extensively purified from porcine pancreas. The porcine enzyme contains fewer lysine residues, sites for autolysis, than the bovine enzyme. The lysine residues have been reductively methylated, producing a product that is resistant to autolysis.[24] It has also been TPCK treated to remove chymotryptic activity. The product is further purified by affinity chromatography and lyophilized from dilute acetic acid. This process yields a highly purified trypsin product that is suitable for proteomics work. This highly purified and chemically stabilized trypsin gives excellent performance in either in-solution or in-gel tryptic digestions. Protein content is based on $E^{1\%}$ = 14.4 at 280 nm.[25] The specific activity is ≥10,000 BAEE units per mg protein.

The product is supplied in a vial containing 20 μg of lyophilized trypsin. The lyophilized product is reconstituted with the Trypsin Solubilization Reagent (1 mM HCl) at the appropriate concentration.

For in-solution digests, prepare the trypsin in the Trypsin Solubilization Reagent at a concentration of 1 mg/ml (20 μl of reagent for the 20 μg vial). This results in a solution containing 1 mg/ml of trypsin in a pH 3.0 solution.

The lyophilized powder retains activity for at least one year if stored unopened at 2–8 °C. The acidic reconstituted solution (pH 3.0) can be stored at 2–8 °C for 2 weeks or at –20 °C for up to 4 weeks. The trypsin solution remains active for at least 3 freeze-thaw cycles.

Solution Digestion Procedure

Trypsin activity is retained in systems containing up to 95% organic solvent.[27] Organic solvents suitable for enzymes are acetonitrile, ethanol, and 1,4-dioxane.[27] Enzymes in an organic solvent often have a higher activity than in aqueous conditions.[28,29,49] In addition, trypsin retains most of its activity in 2.0 M urea, 2.0 M guanidine HCl, or 0.1% SDS.[8-11] The digestion pattern may be influenced by the buffer composition.[30] A peptide such as the oxidized B chain of insulin should be run as a control for all experiments.

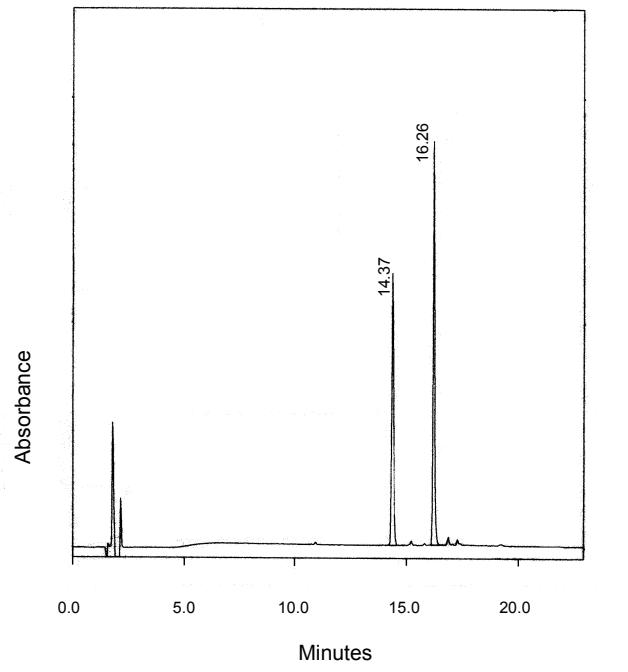
1. Add the prepared trypsin enzyme solution to the sample protein solution. A ratio between 1:20 and 1:100 (w/w) of trypsin to protein substrate is recommended.
2. Incubate the reaction at 37 °C for 2 hours to overnight depending on the enzyme to substrate ratio.
3. Digest is ready to be analyzed by HPLC-MS or MALDI-MS (see Figure 5).

Figure 5.
Trypsin digestion of the oxidized B chain of insulin (Catalog Number I1764)

The sequence of the oxidized B chain of insulin is:

FVNQHLC$_{ox}$GSHLVEALYLVC$_{ox}$GER GFFYTPK A

Tryptic Cleavage Sites



The oxidized B chain of insulin (100 µg) was digested with 2 µg of trypsin for 18 hours at 37 °C in 100 µl of 100 mM Tris-HCl, pH 8.5. A 10 µg aliquot was separated on a Supelco Discovery C18 column (15 cm x 4.6 mm, 5 micron, Catalog Number 504955), using a 20 minute linear gradient from 5–50% B at 1 ml/min with UV detection at 214 nm followed by mass spectrometry.
Solvent A: 0.1% (v/v) TFA in water.
Solvent B: 0.08% (v/v) TFA in acetonitrile.

Retention Time (min)	Mass (Da)	Fragment
14.37	858.4	Gly^{23}-Lys^{29}
16.26	2583.0	Phe^{1}-Arg^{22}

The hydrophilic amino acid Ala^{30} co-elutes with the buffer salts in the injection peak.

During the extensive 18 hour digestion only the expected peptide fragments were generated with no indication of other proteolytic activity. The actual digestion time used can be determined by the user, as under the conditions used for the 18 hour digestion, the cleavage of the substrate peptide is complete in less than 5 minutes.

Appendix
Procedure for In-Gel Digestion

The proteolytic enzymes may also be used for in-gel protein digestions with subsequent identification by mass spectrometry. There are many published procedures for digestions (Asp-N,[31-34] Lys-C,[34-38] and Glu-C,[34,39-41]) in gels or on membranes.[42-48]

Prepare the proteolytic enzyme solution for in-gel digestion (see Table 1). Add the indicated volume of the reconstitution solution (Trypsin Solubilization Reagent for trypsin and Arg-C, and water for Glu-C, Lys-C, and Asp-N). Mix the vial briefly to ensure the enzyme is dissolved. Then add the indicated volume of the 40 mM ammonium bicarbonate with 9% acetonitrile for a 20 μg/ml solution.

Table 1.
Reconstitution of enzymes for in-gel digestion

Enzyme	Amount in vial	Reconstitution solution (volume)	Volume of 40 mM ammonium bicarbonate and 9% acetonitrile	Final Volume	Final Concentration
Trypsin	20 μg	Trypsin Solubilization Reagent (100 μl)	900 μl	1 ml	20 μg/ml
Asp-N	2 μg	water (10 μl)	90 μl	100 μl	20 μg/ml
Glu-C	25 μg	water (125 μl)	1.125 ml	1.25 ml	20 μg/ml
Lys-C	5 μg	water (25 μl)	225 μl	250 μl	20 μg/ml
Arg-C	5 μg	Trypsin Solubilization Reagent (25 μl)	225 μl	250 μl	20 μg/ml

For in-gel digests, prepare a solution by adding 100 μl of Trypsin Solubilization Reagent to one vial of trypsin. Mix the vial briefly to ensure the trypsin is dissolved. Add 900 μl of 40 mM ammonium bicarbonate with 9% acetonitrile solution to the vial and mix. The final concentration of trypsin is 20 μg/ml.

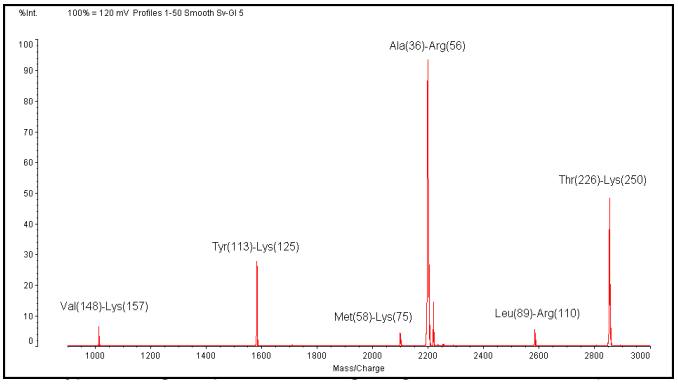
The acidic reconstituted trypsin solution (pH 3.0) can be stored at 2–8 °C for 2 weeks or at –20 °C for up to 4 weeks. The ammonium bicarbonate trypsin solution may be stored either at 2–8 °C for up to 2 weeks or as frozen aliquots for up to 4 weeks. Either trypsin solution retains activity for at least 3 freeze-thaw cycles.

This procedure starts with a Coomassie® Brilliant Blue, SYPRO® Orange, or SYPRO Ruby stained 1D or 2D polyacrylamide gel. For silver stained gels, a gel destaining step different than that used for dye stained gels is required. The ProteoSilver™ Plus Silver Staining Kit (Catalog Number PROTSIL2) is recommended for silver staining prior to enzymatic digestion and MS analysis. It contains destaining solutions for silver stained gels and a procedure for preparing gel slices for enzymatic digestion.

1. Carefully cut the band of interest from a 1D gel or the protein spot from a 2D gel, using a scalpel or razor blade, taking care to include only stained gel. Lift out the gel piece using clean flat nosed tweezers.
2. Place the gel piece in a siliconized Eppendorf® tube or equivalent. A siliconized tube reduces binding of the peptides to the tube surface. If unsure of chemicals leaching from the tube, which could interfere or suppress the MALDI-MS signal, prewash the tube with 100 μl of a 0.1% trifluoroacetic acid in 50% acetonitrile solution and then allow it to dry before use.
   Note: The gel piece may be cut into equal sections of 1 to 1.5 mm in size and the sections may be used in place of the intact piece.
3. Cover the gel piece with 200 μl of 200 mM ammonium bicarbonate with 40% acetonitrile and incubate at 37 °C for 30 minutes. Remove and discard the solution from the tube.
4. Repeat step 3 one more time.
5. Dry the gel piece in a Speed Vac® for 15–30 minutes.

6. Add 20 µl of the enzyme solution (0.4 µg of enzyme) prepared for in-gel digests to the gel sample.
7. Add 50 µl of 40 mM ammonium bicarbonate in 9% acetonitrile solution to the gel sample.
8. Confirm that the gel piece is at the bottom of the tube and covered with liquid.
9. Incubate for 4 hours to overnight at 37 °C.
   Note: A shorter digestion time may be sufficient, but may yield slightly lower sequence coverage.
10. After the incubation, remove the liquid from the gel piece and transfer the liquid to a new labeled tube. This solution contains the extracted digest peptide fragments. If MALDI analysis is to be performed at this step, acidification with TFA prior to matrix addition may be needed.
11. Add 50 µl of a 0.1% trifluoroacetic acid in 50% acetonitrile solution to the gel piece and incubate for 30 minutes at 37 °C.
    Note: This extraction step only increases the peptide yield by about 5%.[48] If the extra 5% is not required for your system, the extraction step can be eliminated and the digest solution from step 10 may then be analyzed.
12. Remove the 0.1% trifluoroacetic acid in 50% acetonitrile solution and combine with the liquid from step 10.
13. The combined digest solution from step 12 is ready for MALDI-MS analysis (see Figure 6).
    Note: If digesting low levels of protein, the sample mixture may need to be concentrated with a ZipTip® before spotting on the MALDI target.

Figure 6.
MALDI analysis of a trypsin in-gel digest of carbonic anhydrase II



A matrix solution of cyano-4-hydroxycinnamic acid was prepared at 10 mg/ml in 70% acetonitrile with 0.1% TFA. The digest solution was desalted using a C_{18} ZipTip and 1.5 µl of the matrix solution was used to directly elute the peptides onto the MALDI target. Data was acquired in linear positive ion mode with a Kratos compact discovery SEQ. The resultant mass spectrum is shown. A database search of the mass list was done using the Mascot database at a tolerance of 300 ppm. The protein was identified as carbonic anhydrase II with a MOWSE score of 102 and a sequence coverage of 42%. The tryptic peptides that were identified by the database are shown above.

Note: A common autolytic fragment observed from a trypsin digest is 842.51 (A_7) m/z produced by arginine cleavage. Other autolytic peptides occasionally detected include the 2239.14 (A_4) and 1045.56 (A_6) m/z. The cited peptide at 2211.10 (A_4) m/z containing an unmodified Lys^{69} is not observed with the Proteomics Grade Trypsin, as it is fully converted to the dimethylated 2239.14 m/z peptide.

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