Protocol

In-Gel Trypsin Digest of Gel-Fractionated Proteins

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INTRODUCTION

This protocol describes a method for in-gel digestion of proteins after fractionation using SDS-PAGE. It is applicable to both one- and two-dimensional polyacrylamide gels of different thicknesses, acrylamide concentrations, and band (spot) sizes. This protocol followed by liquid chromatography/mass spectrometry/mass spectrometry (LC-MS/MS) or matrix-assisted laser desorption/ionization/time of flight/time of flight (MALDI-TOF/TOF) is capable of identifying low femtomole quantities of a protein. High-sensitivity mass spectrometry should be capable of identifying attomole quantities of protein.

RELATED INFORMATION

This is a revised version of "In-Gel Trypsin Digest of Gel-Fractionated Proteins," Appendix 3, in Proteomics: A Cold Spring Harbor Laboratory Course Manual (Link and LaBaer 2009). This protocol was originally adapted from Andrej Shevchenko and Matthias Mann's in-gel digestion protocol (Shevchenko et al. 1996). An updated version was released in 2006 with added information (Shevchenko et al. 2006). SDS-PAGE gels for use in this protocol can be prepared using standard methods such as SDS-PAGE of Proteins (Simpson 2006) or SDS-Polyacrylamide Gel Electrophoresis of Proteins (Sambrook and Russell 2006).

MATERIALS

Reagents

Use the purest chemicals available at all stages of sample preparation, including the gel casting. Wear gloves at all stages to minimize contamination by human epidermal proteins (keratins). Use powder-free gloves to avoid contamination from talcum powder and dust. To avoid contamination from dust and foreign particulates such as keratins, perform all steps in this protocol in a laminar flow hood or in a dust-free environment.

- Acetonitrile
- Ammonium bicarbonate (1 M), freshly prepared
- Digestion solution, freshly prepared and kept on ice
- DTT (Dithiothreitol; 50 mM), freshly prepared
- Formic acid (5%)
- Iodoacetamide (IAA; 100 mM), freshly prepared

IAA is light-sensitive and should be stored in the dark.
Protein sample(s) for fractionation

Stain for gel-bound proteins (see Step 1)

- **Visualize proteins by staining with Coomassie brilliant blue R-250 or G-250, colloidal Coomassie, silver staining, SYRPO Ruby, or reverse staining (zinc-imidazole staining).** If silver staining is used, select a protocol that is compatible with mass spectrometry (*Shevchenko et al. 1996*). Several commercial stains are available that are mass spectrometry-compatible including Pierce’s SilverSNAP, GelCode Blue, and Imperial Protein stain. If silver, fluorescent, or reverse staining is used, then the extensive washing steps prior to reduction and alkylation can be omitted.

**Equipment**

- Camera
- Dry ice
- Glass cleaner (e.g., Windex)
- The Chait laboratory at The Rockefeller University routinely uses Windex to clean spatulas, razor blades, glass plates, and other items used for protein purification protocols and in-gel digest. They have found Windex is highly efficient at removing contaminating proteins such as keratins that notoriously plague in-gel digest without leaving behind chemical contaminants.
- Glass plate
- Ice
- Incubators set to 30°C, 37°C, and 65°C
  
  **The 37°C and 65°C incubations should be performed in incubators that prevent the formation of water condensation at the top of tube.**
- Mass spectrometer (see Step 26)
- Microcentrifuge tubes (500 µL)
- Micropipettor with tips
- Razor blade
- SDS-PAGE gel and electrophoresis apparatus (see Step 1)
- Spatula
- Vacuum evaporator (e.g., Speed-Vac)
- Vortex mixer

**METHOD**

1. Fractionate the protein sample(s) on an SDS-PAGE gel and stain. After staining, rinse the entire gel in water for several hours.

   *The gels can be prepared by standard techniques using 0.1% SDS, e.g., SDS-PAGE of Proteins (Simpson 2006) and SDS-Polyacrylamide Gel Electrophoresis of Proteins (Sambrook and Russell 2006). See Materials for a list of compatible stains.*

2. Photograph the gel and mark the bands to be identified.
3. Using a Windex-cleaned razor blade, spatula, and glass plate, excise the protein bands to be identified. Excise a gel piece of similar size from a non-protein region of the gel and treat identically to the protein band as a control throughout the in-gel digestion protocol and subsequent mass spectrometry analysis. **Cut as close to the protein as possible to reduce the amount of background. Although it is almost impossible to completely eliminate background contaminants, steps should be taken to minimize background contaminants such as human keratins, which can obscure low abundant proteins in the gel. The sequences of common protein contaminants and trypsin should be included in the protein databases used in searching the acquired mass spectra.**

4. Cut the excised gel slice into 1-mm cubes and transfer the gel cubes to a 500-µL microcentrifuge tube. Centrifuge the tube for 1-2 sec to spin the gel slices to the bottom of the tube.

5. For Coomassie-stained gel slices, add 100 µL of 100 mM ammonium bicarbonate/acetonitrile (1:1, v/v) solution and incubate for 30 min with occasional gentle vortexing. For all other stained gel slices, proceed to Step 6. **For Coomassie stained bands, continue washing until the most of the dye is extracted from gel cubes. It is not necessary to completely remove all of the Coomassie dye.**

6. Add 500 µL of acetonitrile and incubate for 10 min. The gel pieces should become opaque and stick together.

7. Prepare a 10 mM DTT, 100 mM ammonium bicarbonate solution (DTT/ammonium bicarbonate).

8. Quickly (1 sec) centrifuge the tube with the gel slices to transfer liquid from the sides of the tube to the bottom. Remove the acetonitrile using a pipettor with a clean pipette tip.

9. Rehydrate the gel slices in DTT/ammonium bicarbonate. Add enough solution to completely cover the gel slices. Add more solution if it is absorbed by the gel pieces. Incubate at 65°C for 30 min. **The DTT reduces the disulfide bonds. Perform the 65°C incubation in an incubator that prevents the formation of water condensation at the top of tube.**

10. Carefully remove the DTT/ammonium bicarbonate and add 500 µL of acetonitrile. Incubate for 10 min at room temperature.

11. Prepare a 50 mM IAA, 100 mM ammonium bicarbonate solution (IAA/ammonium bicarbonate).

12. Quickly (1 sec) centrifuge the tube with the gel slices to transfer liquid from the sides of the tube to the bottom. Remove the acetonitrile using a pipettor with a clean pipette tip.

13. Add the IAA/ammonium bicarbonate to completely cover the gel slices. Incubate for 30 min at 30°C in the dark. Add more solution if it is absorbed by the gel pieces. **This step alkylates cysteine residues and prevents the re-formation of disulfide bonds. The alkylation step adds 57 Da to the mass of cysteine residues. When searching the acquired spectra against a protein database, all cysteine residues will have a fixed modification of 57 Da and the residue mass of cysteine will now be 160 Da (103 Da + 57 Da).**

14. Centrifuge quickly (1 sec) to transfer liquid from the sides of the tube to the bottom. Remove the IAA/ammonium bicarbonate using a clean pipette tip.

15. Add 500 µL of acetonitrile to the gel slices and incubate for 10 min at room temperature.

16. Carefully remove the acetonitrile solution.

17. Add just enough trypsin digestion solution to cover the gel slices. Incubate the gel pieces on ice for 45 min. Add more digestion solution if all the initial solution is absorbed by the gel pieces.
18. Remove the excess digestion solution and add 5–20 µL of 100 mM ammonium bicarbonate solution to keep the gel pieces wet during enzymatic digestion.

19. Incubate overnight at 37°C.
   *Perform the 37°C incubation in an incubator that prevents the formation of water condensation at the top of the tube. For abundant proteins that are detected by Coomassie staining, trypsin digestion for 30 min at 55°C should yield sufficient peptides for mass spectrometric identification (Havlis et al. 2003). For lower-abundance proteins, digestions should proceed overnight.*

20. Using a pipettor and a clean pipette tip, recover the supernatant and transfer into a fresh 500-µL microcentrifuge tube.
   *For abundant proteins that are detected using Coomassie stain, there are typically sufficient peptides recovered to identify the abundant proteins by mass spectrometry without further extraction steps. The non-extracted gel slices can be stored at -20°C for several months.*

21. To recover peptides from low-abundance proteins, add 100 µL of 100 mM ammonium bicarbonate/acetonitrile solution (1:2, v/v) to cover gel slices. Incubate for 15 min at room temperature.
   *For samples with larger volume of gel slices, add extraction buffer such that the approximate ratio of 1:2 between volumes of the digest and extraction is achieved.*

22. Using a pipettor and a clean pipette tip, recover the extraction solution and transfer to the 500-µL microcentrifuge tube with recovered digestion supernatant. The recovered digestion solution and extractions are pooled into the 500-µL microcentrifuge tube.

23. Freeze the pooled supernatants on dry ice.

24. Lyophilize the extracted peptides to near dryness. DO NOT OVERDRY! Stopping when there is 1-2 µL of liquid is ideal.
   *Lyophilized extracts can be stored at -20°C.*

25. Resuspend peptides in 2-20 µL of 5% formic acid.
   *It is estimated that peptide recovery at the picomole level is on average 70%-90% compared to in-solution digest (Shevchenko and Shevchenko 2001; Havlis et al. 2003; Havlis and Shevchenko 2004).*

26. Analyze using LC-MS/MS or MALDI-TOF/TOF.

**REFERENCES**


Caution

Acetonitrile

Acetonitrile is very volatile and extremely flammable. It is an irritant and a chemical asphyxiant that can exert its effects by inhalation, ingestion, or skin absorption. Treat cases of severe exposure as cyanide poisoning. Wear appropriate gloves and safety glasses. Use only in a chemical fume hood. Keep away from heat, sparks, and open flame.

Caution

Ammonium bicarbonate

Ammonium bicarbonate, NH₄HCO₃, may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses and use in a chemical fume hood.

Caution

Coomassie brilliant blue (CBR)

Coomassie brilliant blue (CBR) may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses.

Caution

DTT (Dithiothreitol)

DTT (dithiothreitol) is a strong reducing agent that emits a foul odor. It may be harmful by inhalation, ingestion, or skin absorption. When working with the solid form or highly concentrated stocks, wear appropriate gloves and safety glasses and use in a chemical fume hood.

Caution

Dry ice (Carbon dioxide; CO₂)

CO₂ (carbon dioxide; dry ice) in all forms may be fatal by inhalation, ingestion, or skin absorption. In high concentrations, it can paralyze the respiratory center and cause suffocation. Use only in well-ventilated areas. In the form of dry ice, contact with carbon dioxide can also cause frostbite. Do not place large quantities of dry ice in enclosed areas such as cold rooms. Wear appropriate gloves and safety goggles.

Caution

Formic acid (HCOOH)
Formic acid (HCOOH) is highly toxic and extremely destructive to tissue of the mucous membranes, upper respiratory tract, eyes, and skin. It may be harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses (or face shield) and use in a chemical fume hood.

**Caution**

**Iodoacetamide (C₂H₄INO)**

Iodoacetamide (C₂H₄INO) can alkylate amino groups in proteins and can therefore cause problems if the antigen is being purified for amino acid sequencing. It is toxic and harmful by inhalation, ingestion, or skin absorption. Wear appropriate gloves and safety glasses and use only in a chemical fume hood. Do not breathe the dust.

**Recipe**

**DTT (Dithiothreitol; 50 mM)**

> DTT (Dithiothreitol) (VWR EM-3860)

Add 7.7 mg of DTT (FW 154) to a 1.5-mL microcentrifuge tube. Add 1 mL of Milli-Q H₂O. Prepare fresh. Discard unused DTT solution.

**Recipe**

**Digestion solution**

- 100 mM ammonium bicarbonate
- 0.5 mM CaCl₂
- 15 ng/µL modified sequencing-grade trypsin (Promega V5111)
- 5% (v/v) acetonitrile

Prepare the digestion solution fresh and keep it on ice. Discard unused digestion solution.

**Recipe**

**Iodoacetamide (100 mM)**

> Iodoacetamide (IAA) (Sigma I-1149)

Add 18.5 mg IAA (FW 184) to a 1.5-mL microcentrifuge tube. Add 1 mL of Milli-Q H₂O. Store in the dark until ready to use. Prepare fresh. Discard unused IAA solution.