Protocol

In-gel Digestion and Extraction of Proteins Protocol

Modified from University of Wisconsin – Biotechnology Center Protocol

Introduction

Polyacrylamide gel electrophoresis is a technique used to separate proteins for downstream analysis, including mass spectrometry, leading to their identification and structural analysis. For mass spectrometry and other analysis to give accurate results, proteins must first be extracted and prepared using an appropriate in-gel protein digestion procedure. During in-gel digestion, the protein of interest is excised from the coomassie or silver-stained polyacrylamide gel. Then, SDS is removed with ammonium bicarbonate/acetonitrile. The fragments are then rehydrated with digestion enzyme in buffer. Finally, proteins are extracted and ready for further analysis. This protocol describes a complete digestion for proteins of interest.

Materials

- Previously harvested protein bands
- dH_2O
- 50% Acetonitrile
- Acetonitrile
- Dithiothreitol (GoldBio Catalog # DTT)
- Iodoacetamide
- Ammonium Bicarbonate [$(NH_4)HCO_3$]
- Trifluoroacetic Acid (TFA)

For Blue Stain:

- Coomassie Brilliant Blue G-250 (GoldBio Catalog # C-460) or
- Comassie Brilliant Blue R-250 (GoldBIo Catalog # C-461)
- 50% Methanol

For Silver Stain:

- Sodium Thiosulfate [Na2S2O3]
- Potassium Ferricyanide [K3Fe(CN)6]

Method

Gel fragment preparation

1. Excise protein bands/spots of interest from polyacrylamide gel. Cut each into 1 mm pieces and place into a low binding centrifuge tube.

Note: Cut out a piece from a protein-free region of gel to run in parallel as a control.

Note: In case of faint silver-stained spots, particles can be pooled. However, should not pool more than three particles.

2. Wash gel pieces twice with copious dH_2O.

Destaining (Choose a or b and proceed to step 1)

a. For coomassie blue, destain two times for 5 minutes or until colorless with 200 µl 100mM $(NH_4)HCO_3$/50% Methanol (discarding supernatants). Dehydrate for 5 minutes with 200 µl 25mM Ammonium bicarbonate $(NH_4)HCO_3$/50% Acetonitrile, then once more for 100% acetonitrile. Remove the solution and discard. The gel pieces will shrink and become white.

b. For nondestructive silver, destain twice for 2 minutes or until colorless with 200 µl of freshly prepared 1:1 solution of 100mM Sodium Thiosulfate [$Na_2S_2O_3$] and 30mM Potassium Ferricyanide [$K_3Fe(CN)_6$]. Stop the reaction and wash out the silver ions twice for 2 minutes with 500 µl of dH2O. Dehydrate for 5 minutes with 200 µl 25mM Ammonium Bicarbonate $(NH_4)HCO_3$/50% Acetonitrile, then once more for 30 seconds with 100% Acetonitrile. The gel pieces will shrink and become white.

1. Dry gel particles for 2 minutes in a vacuum centrifuge.

Reduction and alkylation
Proteins that have been separated by two-dimensional gel electrophoresis are already reduced and alkylated, so these steps may be omitted.

1. Prepare fresh:
   a. 25mM Dithiothreitol, in 25mM Ammonium Bicarbonate (NH_4HCO_3).
   b. 55mM Iodoacetamide, in 25mM Ammonium Bicarbonate.

2. Rehydrate gel pieces in 50 µl of prepared 25mM DTT.

3. Incubate to reduce the proteins for 20 minutes at 56°C.

4. Cool the gel fragments to room temperature, pipette off any residual liquid and add 50 µl of 55mM Iodoacetamide.

5. Incubate to alkylate the proteins in the dark for 20-30 minutes at room temperature.

6. Remove liquid and wash gel pieces with ≥ 20 volumes of dH_2O water (~400 µl) for 30 seconds to remove residual Iodoacetamide. Dehydrate gel pieces for 5 minutes with 200 µl 25mM $(NH_4)HCO_3$/50% Acetonitrile.

7. Remove liquid and dehydrate gel pieces again for 30 seconds in 100% Acetonitrile.

8. Dry gel particles for 2 minutes in a vacuum centrifuge.

Digestion

1. Prepare fresh trypsin solution (10 ng/µl in 25mM Ammonium Bicarbonate/3% Acetonitrile at pH ~8.5).

Note: Do not add more solution than can be absorbed by the gel particles.

2. Rehydrate with 40 µl trypsin solution for 5 minutes at room temperature.

3. Overlay the rehydrated gel fragments with a minimum amount of 25mM $(NH_4)HCO_3$ to keep them immersed. Incubate for 16-24 hours (or overnight, if desired) at 37°C.

Peptide recovery

1. Extract digested peptides with 50 µl dH_2O/1% TFA by vortexing (maximum speed) for 10 minutes at room temperature. Transfer solution to a new low-binding tube.

2. Perform an addition extraction with 80 µl of 70% acetonitrile/25% H_2O/5% TFA.

3. Dry peptide solution completely in a vacuum centrifuge (1-2 hours).

4. Reconstitute peptides in 20 µl dH_2O/0.1% TFA by incubating for 5 minutes at room temperature with intermittent vortexing.

5. Sample is ready for LC-based analysis.

Tips

- Low concentrations of ammonium bicarbonate in the digestion buffer will give the best results for analysis such as MALDI-MS.
- Use silanized tubes to prevent sample loss.

- Low amounts of single-component detergents may improve peptide recovery.

Associated Products

- Dithiothreitol (GoldBio Catalog # DTT)
- Coomassie Brilliant Blue R-250 (GoldBio Catalog # C-461)
- Coomassie Brilliant Blue G-250 (GoldBio Catalog # C-460)
- Blazin' Blue™ Protein Gel Stain (GoldBio Catalog # P-810)

References

Jiménez, C., Huang, L., Qiu, Y., and Burlingame, A. (1998). In-Gel Digestion of Proteins for MALDI-MS Fingerprint Mapping. *Current Protocols in Protein Science*. Doi:10.1002/0471140864.ps1604s14.

University of Wisconsin–Madison Biotechnology Center. (n.d.). In gel digestion of proteins. Retrieved May 18, 2018, from https://www.biotech.wisc.edu/services/massspec/protocols/ingelprotocol.