In-gel Digestion of Proteins

(Modified from University of Wisconsin – Biotechnology Center protocol)

Gel fragment preparation

- Excise protein bands. Cut each into 1 mm pieces. Place into a low-binding centrifuge tube.
- Wash gel pieces twice with copious dH_2O (~200 µl) for 30 seconds each to wash out acetic acid.

Destaining

- **Coomassie Blue** – (Coomassie Brilliant Blue G-250 or Coomassie Brilliant Blue, R-250) Destain two times for 5 minutes or until colorless with 200 µl 100mM $(NH_4)HCO_3$/50% Methanol (discard supernatants). Dehydrate for 5 minutes with 200 µl 25mM $(NH_4)HCO_3$/50% Acetonitrile, then once more for 30 seconds in 100% Acetonitrile. Remove the solutions and discard. The gel pieces shrink and become white.
- **Non-Destructive Silver staining** – 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 silver ions twice for 2 minutes with 500 µl of Millipore water. Dehydrate for 5 minutes with 200 µl 25mM $(NH_4)HCO_3$/50% Acetonitrile, then once more for 30 seconds in 100% Acetonitrile. The gel pieces shrink and become white.
- **Sypro Ruby staining** – No destaining necessary. Dehydrate for 5 minutes with 200 µl 25mM $(NH_4)HCO_3$/50% Acetonitrile, then once more for 30 seconds in 100% Acetonitrile. Remove the solutions and discard. The gel pieces shrink and become white.
    - Dry gel particles for 2 minutes in a vacuum centrifuge.

Reduction and Alkylation

1. Prepare fresh:
    - 25mM Dithiothreitol (DTT; GoldBio Catalog # DTT) in 25mM Ammonium Bicarbonate ($(NH_4)HCO_3$), and
    - 55mM Iodoacetamide in 25mM Ammonium Bicarbonate.
2. Rehydrate gel pieces in 50 µl of prepared 25mM DTT. Reduce the proteins for 20 minutes at 56°C.
3. Cool the gel fragments to room temperature, pipet off any residual liquid and add 50 µl of 55mM Iodoacetamide. Alkylate the proteins for 20-30 minutes at room temperature in the dark.
4. 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.

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

6. 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, pH ~8.5).

2. Rehydrate with 40 µL Trypsin solution and incubate for 4-5 hours (or overnight, if desired) at 37°C.

3. Overlay the rehydrated gel particles (if necessary) with a minimum amount of 25mM $(NH_4)HCO_3$ to keep them immersed throughout the digestion.

Peptide Recovery

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

2. Perform an additional 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.

References

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