In-gel and In-solution Tryptic Digestion

Equipment and Materials:

Ammonium Bicarbonate (Fluka #09830); Acetonitrile, HPLC Grade (Fisher #A998-4); Dithiothreitol (DTT) (Sigma #43815); Eppendorf tubes, 1.5 mL (Eppendorf #022363204) Eppendorf tubes, 0.5 mL (Eppendorf #022363611); Formic acid, 88% (J.T. Baker #0128-01) Iodoacetamide (Sigma #I1149); Trypsin, Sequencing Grade (Promega #V5111); Water, Milli-Q system; Assorted pipets and sterile pipette tips; Eppendorf Tube Vortex Shaker with Multi-tube Attachment; Disposable scalpel, sterile methanol wash bottle; Speedvac Vacuum Centrifuge; Ice bucket

Procedure:

Preparation of reagents

1. Prepare 100 mM solution of ammonium bicarbonate (79.056 g/mol) by dissolving 3.95 g in 500 mL Milli-Q water. Check that pH is between 8-9. Solution is good for ~1 month at 4°C.
2. Prepare 0.1% solution of formic acid by pipetting 11.4 µL 88% formic acid into 10 mL Milli-Q water. Check that pH is between 2-3. Solution is good for ~3 months 4°C.
3. Prepare 500 ng/uL aliquots of trypsin from stock by dissolving one vial (20 µg enzyme) with 40 µL of supplied resuspension buffer. Distribute 10 µL aliquots into 0.5 mL tubes. Store at -80°C until use.
4. For in-gel procedure, prepare a fresh solution of 10 mM DTT (154 g/mol) in Milli-Q water. Make enough DTT for roughly 100 µL to be applied to each gel band. Usually 1 mL is enough.

5. For in-gel procedure, prepare a fresh solution of 55 mM iodoacetamide (184.96 g/mol) in Milli-Q water. Make enough iodoacetamide for roughly 100 µL to be applied to each gel band. Usually 1 mL is enough. Keep iodoacetamide solution in the dark.
6. Prepare a fresh solution of 50% acetonitrile/5% formic acid in glass by combining 5 mL of acetonitrile with 4.5 mL of Milli-Q water and 500 µL of formic acid.

In-gel Procedure

1. Prepare a clean, methanol-rinsed glass cutting surface.
2. Using a sterile scalpel, excise gel bands of interest, minimizing the removal of non-stained regions of polyacrylamide. Rinse the scalpel with methanol between gel bands.
3. Dice individual gel bands into ~1 mm pieces (just big enough such that pieces cannot be sucked up into a pipette tip) in small droplets of ammonium bicarbonate to keep bands wet. Separate the gel band regions. Rinse the scalpel with methanol between gel bands. Carefully transfer pieces from individual gel band regions into separate 1.5 mL tubes using the scalpel as a scoop.
4. Add just enough ammonium bicarbonate to cover the gel material (75-100 µL). Shake on a vortex shaker for 5 minutes.
5. Remove and discard excess liquid.
6. Repeat steps 4 and 5.
7. Dry down bands in a Speedvac. Make sure the interior of the Speedvac has been fully wiped down with methanol.
8. Add just enough DTT (75-100 µL) to cover the gel material. React for 1 hour on a vortex shaker.

9. Remove and discard excess liquid.
10. Add just enough iodoacetamide (75-100 µL) to cover the gel material. React for 45 minutes on a vortex shaker in the dark. A piece of aluminum foil can be used to cover the shaker.
11. Wash with just enough ammonium bicarbonate to cover gel material (75-100 µL). Shake on a vortex shaker for 5 minutes.
12. Remove and discard excess liquid.
13. Add just enough 100% acetonitrile to cover the gel pieces (75-100 µL). Shake on a vortex shaker for 5 minutes.
14. Remove and discard excess liquid.
15. Dry down the gel pieces in a Speedvac.
16. Prepare 12.5 ng/µL of trypsin from 500 ng/µL aliquots by combining 10 µL of trypsin with 390 µL of ammonium bicarbonate.
17. Cover the gel pieces in trypsin solution and let the tubes sit on ice for 45 minutes.
18. Remove and discard excess liquid.
19. Add just enough ammonium bicarbonate to cover the gel pieces. Shake on a vortex shaker for 8-12 hours.
20. Remove and discard excess liquid.

    Note: At this point the extraction procedure begins. Do not discard any wash solutions, as these contain your peptides. Collect and pool the elute from each gel band tube into a new tube suitably labeled.

21. Dehydrate with just enough 50% acetonitrile/5% formic acid to cover the gel pieces. Shake on a vortex shaker for 15 minutes.
22. Remove and collect solution.

23. Rehydrate with just enough ammonium bicarbonate to cover the gel pieces. Shake on a vortex shaker for 15 minutes.
24. Collect and pool solution.
25. Dehydrate with just enough 50% acetonitrile/5% formic acid to cover the gel pieces. Shake on a vortex shaker for 15 minutes.
26. Collect and pool solution.
27. Rehydrate with just enough ammonium bicarbonate to cover the gel pieces. Shake on a vortex shaker for 15 minutes.
28. Do not remove the solution. Dehydrate with 3x the volume used in step 27 of 100% acetonitrile.
29. Collect and pool solution.
30. Dry down pooled solutions in a Speedvac to near-dryness.
31. Reconstitute in the appropriate volume (25-50 µL) 0.1% formic acid.
32. Digests can be stored in a -20°C freezer prior to analysis.

In-solution Procedure

1. Check the pH of the sample. If the solution is basic, continue to step 2. If the solution is acidic, lyophilize to near dryness and reconstitute in 100 mM ammonium bicarbonate to a reasonable volume (<100 µL).
2. Calculate the moles of cysteines in the sample. Make a fresh solution of DTT (154.25 g/mol) in water such that adding 1 µL DTT gives 20x the molar amount of cysteine. For example, if you have 10 µL of a 1 pmol/µL solution of a protein with 3 cysteines (30 pmol cysteines), make a 0.6 nmol/µL solution of DTT and add 1 µL. Let react for 1 hour at 37°C.

3. Calculate the concentration of DTT in solution. Make fresh solution of iodoacetamide (184.96 g/mol) in water such that adding 1 µL iodoacetamide makes the concentration of iodoacetamide 3x the concentration of DTT. (For example, after the example reaction with DTT shown in step 2, the concentration of DTT in solution is 54.5 pmol/µL, and the concentration of iodoacetamide in solution should be 163.6 pmol/µL. To obtain this concentration after adding 1 µL iodoacetamide to yield at total volume of 12 µL, we need to make a 1.9 nmol/µL solution of iodoacetamide). Let the reaction go for 1 hour in the dark.
4. Calculate the amount of trypsin to add for a ratio of 1:20 or 1:15 enzyme:protein based on weight. For example, if you have 10 pmol of a 40 kDa protein, you should add 20 ng trypsin based on a 1:20 ratio.
5. Let react 6-8 hours at 37°C.
6. Quench with 1 µL of 88% formic acid. The pH should be between 2-3. If the solution is still basic, add another 1 µL of 88% formic acid.
7. The digest can be stored in a -20°C freezer prior to analysis.

1.0 Expected Outcome/Data:

Trypsin cleaves after arginine and lysine residues to yield peptides typically 5-20 in length and typically with 2-3 charges when the analysis is performed by electrospray, making them ideal for analysis by C18 RP-LC-MS/MS under CAD conditions. However, missed cleavages are common, especially when consecutive basic residues are present.

7.0 References:

Schevchenko, A., Wilm, M., Vorm, O., Mann, M. (1996) *Anal. Chem*. **68**, 850-858