In-Gel Trypsin Digestion

Reagents: (prepare freshly right before the digestion)

(Use HPLC grade solvents, highest possible grade reagents and MilliQ water for all preparations)

Wash solution: 50% methanol and 5% acetic acid.

25 mM NH_4HCO_3 in 50% acetonitrile (ACN)

25 mM NH_4HCO_3

5% formic acid in water

5% formic acid in 50% ACN

12.5 ng/μl trypsin in 25 mM NH_4HCO_3 (Sequencing Grade Modified Trypsin, Promega Cat# V5111)

1.5-ml microcentrifuge tubes: Low protein binding, siliconized tubes are recommended. Rinse tubes in USP-grade methanol and dry before the use.

Procedure:

1. Excise protein spot/band (from SYPRO Ruby, Coomassie, or MS compatible silver stained gels), cut gel slice into small pieces (~1 mm^3) and place them in a 1.5-ml microcentrifuge tube (siliconized, methanol washed).
2. Add 200 μl Wash solution to the gel pieces for 4 hr to overnight. Use gel loading pipet tips to remove the supernatant and discard. Repeat once for 2-3 hr.
3. Add 200 μl of 25 mM NH_4HCO_3 in 50% ACN and vortex 10 min. Repeat this step once or twice.
4. Dry the gel pieces in a Speed Vac. (~30 min)
5. Reduction and Alkylation (Optional, although this has been done before 2^{nd} dimension PAGE, for better digestion and recovery this step is recommended).
   a.) Add 40 μl (or enough to cover) of 10 mM DTT in 25 mM NH_4HCO_3 and incubate at 56^oC for 1 h. Remove and discard supernatant. Let the tube cool to room temp.
   b.) Add the same volume of 55 mM iodoacetamide in 25 mM NH_4HCO_3. Incubate at room temp for 45 min in the dark with occasional vortex. Remove and discard supernatant.
   c.) Wash with ~100 μl of 25 mM NH_4HCO_3 and vortex 10 min. Remove and discard supernatant.
   d.) Dehydrate gel pieces with ~100 μl of 25 mM NH_4HCO_3 in 50% ACN, vortex 5 min. Remove and discard supernatant.
   e.) Repeat steps c. and d. for one more time.
   f.) Dry the gel in a Speed Vac.

6. Add 25 μl (or enough for gel to absorb) of 12.5 ng/μl Trypsin in 25 mM NH_4HCO_3 to the dry gel pieces, wait for ~15 min until the trypsin solution has been absorbed by the gel pieces, remove access solution and discard. Add a minimum amount of 25 mM NH_4HCO_3 without trypsin to keep gel pieces immersed throughout digestion. Incubate at 37^{o}C for 12-16 h.
7. To recover the peptides from the gel.
   a.) Add 30 μl of 5% formic acid in 50% ACN, agitate at rt for 30-60 min. Remove the solution into a clean siliconized tube.
   b.) Add 15 μl of 5% formic acid, incubate at rt for 10 min, and then add 15 μl of 100% ACN, agitate for 30-60 min.
   c.) Combine two extract solutions and dry in a Speed Vac.
8. Dissolve the dry pellet in ~10-20 μl of 0.1% formic acid, and store the peptide solution at -20^{o}C.

Reference:

1. Shevchenko, A., Wilm, M., Vorm, O., Mann, M. 1996. Mass spectrometric sequencing of proteins in silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850-858
2. Jiménez, C. R., Huang, L., Qiu, Y., and Burlingame, A. L. 2003. In-gel digestion of proteins for MALDI-MS fingerprint mapping. in Current Protocols in Protein Science, John E. Coligan, Ben M. Dunn, David W. Speicher, and Paul T. Wingfield (eds.), John Wiley & Sons, Inc. Unit 16.4