In-gel digestion of proteins for peptide fingerprint mapping

Polyacrylamide gel electrophoresis is a widely used technique to separate proteins from biological samples. Moreover, the development of two-dimensional (2D) gel electrophoresis has provided a tool for differential protein display, which allows for the quantitative analysis of many (~500-1000) of proteins simultaneously. Complex biological questions can now be approached by analyzing differences in the 2D gel patterns between control and experimental states.

While high sensitivity staining methods could visualize proteins at increasingly low levels, originally protein identification was limited by the sensitivity of Edman sequencing. However, in the last few years mass spectrometry has become established as a viable and more sensitive alternative, with the development of ESI-MS and MALDI-MS. Peptide mass fingerprinting uses the peptide masses obtained by digestion to search the protein and DNA databases, to find proteins that show a similar theoretical digest pattern (see e.g., Shevchenko et al. 1996a and b).

In the protocol below, we describe the in-gel digestion procedure, as it is routinely performed in our laboratory, for peptide mass mapping of picomole to subpicomole quantities of protein derived from coomassie- or silver stained polyacrylamide gels. First, SDS is removed from the gel prior to digestion, in several ammonium carbonate/acetonitrile washing steps. The excised gel pieces are subsequently dried and rehydrated with enzyme in buffer. Alternatively, the washed gel pieces may be reduced and S-alkylated prior to rehydration with enzyme. After digestion, the peptides are extracted from the gel and mass analyzed.

Materials

25 mM NH_4HCO_3 in 50 % acetonitrile
25 mM NH_4HCO_3, pH 8
5 % TFA/ 50 % acetonitrile
0.1 mg/ml trypsin (sequence grade, Promega) in 25 mM NH_4HCO_3, pH 8
10 mM dithiotreitol in 25 mM NH_4HCO_3
55 mM iodoacetamide in 25 mM NH_4HCO_3

Generally, trypsin is the protease of choice for peptide mass fingerprinting, because of its reliability and its substrate specificity, yielding peptides with C-terminal basic residues (Arg and Lys), which facilitates ionization and subsequent mass spectrometric sequencing. Dissolve trypsin just before use in ice-cold buffer (to reduce auto-proteolysis).

In-gel digestion procedure

1. Excise protein bands/spots of interest from the gel and cut each gel piece into small particles (~1 mm x 1 mm) using a scalpel and place into a 0.65 ml siliconized tube (PGC scientific). Also cut out a gel piece from a protein-free region of the gel, for a parallel control digestion to identify trypsin autoproteolysis products. A small gel particle size facilitates the removal of SDS (and coomassie) during the washes, and improves enzyme access to the gel. For a silver-staining protocol compatible with mass spectrometry, see Shevchenco et al. (1996a).

2. Add ~100 ml 25 mM NH_4HCO_3 in 50 % acetonitrile (or enough to immerse the gel particles) and vortex for 10 min. Use gel-loading pipet tips to remove the solution (pale blue in case of coomassie staining) and discard. Repeat this wash/dehydration step up to ~2-3 times. At this point, the gel slices shrink and become white. This visual criterium should be used to determine whether or not additional washes should be performed.

3. Dry the gel particles for ~15 min in a vacuum centrifuge.

4. Optional reduction and alkylation. Add 10 mM dithiotreitol in 25 mM NH_4HCO_3, enough to cover the gel pieces and reduce for 1 hr at 56 °C. Cool to room temperature and replace the DTT solution by roughly the same volume 55 mM iodoacetamide in 25 mM NH_4HCO_3. Incubate for 45 min at room temperature in the dark with occasional vortexing. Wash the gel pieces with ~100 ml 25 mM NH_4HCO_3 for 10 min while vortexing, dehydrate with ~100 ml 25 mM NH_4HCO_3 in 50 % acetonitrile and rehydrate again with ~100 ml 25 mM NH_4HCO_3 and dehydrate again. Remove the liquid phase and dry the gel pieces in a vacuum centrifuge.

5. Rehydrate the gel particles in 25 mM NH_4HCO_3, pH 8, containing 0.05 - 0.1 mg/ml trypsin by vortexing for 5 min. Do not add more solution than the amount that can be absorbed by the gel particles, otherwise a lot of trypsin autolysis will occur. The enzyme-to-substrate ratio employed for in-gel digestions is greater (> 1:10) than for in-solution digestions due to the hindered enzyme access to the protein substrate in the gel. Moreover, the relative low salt concentration of 25 mM is used to reduce the possibility of subsequent salt interference with ionization in the mass spectrometer. This concentration may be increased if poros microtips or ziptips are subsequently used for desalting.

6. If neccessary, overlay the rehydrated gel particles with a minimum amount of 25 mM NH_4HCO_3, pH 8, to keep them immersed throughout digestion.

7. Incubate 12-16 hours at 37 °C.

8. To recover the peptides from the gel particles, perform ~3 extractions. For the first extraction, add 2 volumes of water and vortex for 10 min. For subsequent extractions, add 5 % formic acid/50 % acetonitrile. Use gel-loading

tips to remove the peptide solution after each extraction and collect in a siliconized tube. The siliconized micro centrifuge tubes and the high formic acid concentration are used to minimize adsorptive sample loss. TFA (5 %) may be used as an alternative for formic acid if MALDI-MS is used.

9. Concentrate the recovered peptides by reducing the final volume of the extracts to ~10 ml in a vacuum centrifuge and add 5 ml 5 % Formic acid/ 50 % acetonitrile.

10. Store the recovered peptides at -20 °C in slick tubes.

References

Fenselau, C. 1997. MALDI-MS and strategies for protein analysis. *Anal. Chem*. 661A-665A.

Jungblut, P. and Thiede, B. 1997. Protein identification from 2-DE gels by MALDI mass spectrometry. M*ass Spectrom. Rev*. **16**:145-162.

Patterson, S.D. and Aebersold, R. 1995. Mass spectrometric approaches for the identification of gel-separated proteins. *Electrophoresis* **16**:1791-1814.

Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. 1996a. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem*. **68**:850-858.

Shevchenko, A., Jensen, O.N., Podtelejnikov, A.V., Sagliocco, F., Wilm, M., Vorm, O., Mortensen, P., Shevchenco, A., Boucherie, H. and Mann, M. 1996b. Linking genome and proteome by mass spectrometry: Large scale identification of yeast proteins from two-dimensional gels. *Proc. Natl. Acad. Sci. USA* **93**:14440-14445.