Tryptic digestion directly in polyacrylamide gel slices (05/05).

This protocol is designed for the direct digestion of proteins in polyacrylamide gels. Direct digestion in the gel band eliminates transfer of the protein of interest to a membrane and increases recovery of peptides. The digests produced by this protocol are amenable to direct analysis by capillary ESI-LC/MS (MS/MS) without fractionation or other clean-up. We believe any gel band that is detectable by Coomassie blue represents sufficient protein for sequence analysis using this method. In addition, bands not detectable by Coomassie but detectable by silver stain (Vorum method; no kits) or any of the fluorescent stains may often contain enough protein for analysis. In general the success rates are as follows: Coomassie (>99%), mid-silver (fluorescent) up to Coomassie (~85%), barely detectable by silver (fluorescent) up to mid-silver (fluorescent) (~60%). While too little protein in the gel band can be a problem, other common problems (reasons for failure) are heavy post-translational modifications (glycosylation or lipids) that block enzymes or lack of tryptic sites (few Lys or Arg). Also, when little protein is present, keratin contamination in/on the gel can interfere and careful efforts should be made to minimize it. This digestion protocol is based on the published method of Mann (Anal Chem, 1996, 68:850-858) for the digestion of silver stained gels for analysis by nanospray mass spectrometry with modifications based on personal observations made in our laboratory. Although the protocol is described for a modified trypsin, chymotrypsin, Glu-C and Lys-C endoproteases may also be used, keeping in mind that these enzymes may contribute higher amounts of autolysis peptides to the sample.

A. Reagents (All reagents prepared fresh).

1. Destain: 50% methanol/5% acetic acid in water.
2. 100 mM ammonium bicarb: 100 mM ammonium bicarbonate in distilled water. (0.158g/20mL)
3. 50 mM ammonium bicarb: 50 mM ammonium bicarbonate in distilled water. (0.079g/20mL)
4. Acetonitrile.
5. 10 mM DTT: 1.5 mg/mL in 100 mM ammonium bicarb.
6. 50 mM iodoacetamide: 10 mg/mL in 100 mM ammonium bicarb.

7. Trypsin solution (on ice): 20 ng/μL Promega sequencing grade modified trypsin (catalog number V511, 20 μg, porcine) in 50 mM ammonium bicarbonate.
8. Extraction solution 1: 5% formic acid in water.
9. Extraction solution 2: 5% formic acid in 50% acetonitrile.

B. Eppendorf tubes.

Siliconized 0.5 and 1.5 mL tubes purchased from Marsh Biomedical Products Inc. All tubes are rinsed in ethanol, water, ethanol and air-dried (covered to keep dust off) prior to use.

C. Procedure.

At all times monitor the gel pieces to ensure they stay in the original tube and do not stick to the pipet tip and get transferred to the extraction tube. The microfuge steps help ensure the gel pieces (fragments) are on the bottom of the tube before transfers of liquid. When removing destain, washes, alkylation and reduction solutions, the same pipet tip may be used without cross contamination. The tip is changed at each different step in the process though. All tips are also rinsed with liquid before the first use. During the extraction step, the same pipet tip is used for all steps (one per sample).

Day 1

1. Cut bands from gel as closely as possible. Divide into ~1mm pieces.
2. Destain the bands in 0.5 mL destain for 2 h.
3. Remove destain and replace with 0.5 mL destain for 1 h.
4. Remove the destain (discard), and dehydrate gel slices in 200 μL acetonitrile for about 5 min. Remove acetonitrile. Repeat.
5. Evaporate gel pieces completely in SpeedVac (2 to 3 min).
6. Reduce the gel pieces in 30 μL 10 mM DTT for 0.5 h at room temperature.
7. Microfuge and remove DTT solution.
8. Alkylate in 30 μL 50 mM iodoacetamide at room temperature for 0.5 h.
9. Microfuge and remove iodoacetamide solution.
10. Wash with 100 μL 100 mM ammonium bicarb for 10 min and remove.

11. Dehydrate gel slices in 200 µL acetonitrile approximately 5 min. Remove acetonitrile.
12. Rehydrate gel pieces in 200 µL 100 mM ammonium bicarb for 10 min.
13. Microfuge and remove ammonium bicarb.
14. Dehydrate gel slices in 200 µL acetonitrile approximately 5 min. Remove acetonitrile. Repeat.
15. Take gel pieces to complete dryness in SpeedVac (2 to 3 min).
16. Prepare trypsin. 20 µg of Promega trypsin in 1000 µL ice cold 50 mM ammonium bicarb (trypsin concentration = 20 ng/µL). Keep ice cold.
17. Add 30-50 µL of the trypsin solution to cover the gel pieces and rehydrate on ice for 30 min.
18. Microfuge and remove any excess trypsin solution and add 5-20 µL 50 mM ammonium bicarb. React overnight (minimize time from evening to early morning) at 37 ^{o}C.

Day 2

19. Extract with 10 uL exteraction solution #1 (volume may be up to 30 µL if large amount of gel). Incubate 10 min., microfuge and take off supernatant to a cleaned 0.5 mL microfuge tube.
20. Extract the peptides by adding 10 µL extraction solution #2. Incubate for 10 min and collect the extract in the 0.5 mL Eppendorf tube. Repeat (all extracts to the same tube).
21. Evaporate the sample to 1 µL (try not to go to complete dryness) then reconstitute to 20 µL total volume with 1% acetic acid for LC-ESI MS (MS/MS) analysis. Store at -20°C.
22. For analysis, anywhere from 0.1 – 10 µL of solution will be directly loaded onto a 75 um x 8 cm (pulled tip) packed with C18.

Notes:

1) Reduction and alkylation are not absolutely necessary for Coomassie stained bands. The peptide yield and protein coverage are greater however if these procedures are used, and

these aspects are important for silver-stain levels of protein or for post-translational modifications where sequence coverage is important.

Tryptic digestion of solution samples (05/05).

If the sample arrives as a pellet (or undergoes acetone precipitation), it will be necessary to redissolve the pellet in 10 μL 1% SDS or 6M urea. SLOWLY dilute using 50 mM ammonium bicarbonate to 0.1% SDS or 1M urea. Sonication can be used on either SDS- or urea-containing samples to aid in redissolving the pellet. Heat may be used on SDS-containing samples to aid in resolubilization, but not on urea-containing samples. For solution sample (or after dissolving pellet), add 5 μL DTT solution for 30 min at RT to reduce followed by 5 μL Iodoacetamide solution for 30 min at RT to alkylate (generally 100-200 μL of protein mixture). Then add 1 μg modified trypsin overnight at RT (add second 1ug over second night at RT if the protein solution contains detergent, urea, FLAG, etc.). At the end of digestion add 10% acetic acid by volume. The peptides should be desalted and concentrated using a reverse phase column. If the sample contained detergent (SDS, NP40), then after desalting the peptides should be cleaned by strong cation exchange.