Procedure for Preparation of Coomassie G-250-stained 1-Dimensional Gel Band for In-gel Trypsin Digestion and MALDI-TOF MS Analysis

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1. Excise gel band and place into a 1.5 mL **natural (not colored) Eppendorf Safelock microcentrifuge tube**. Label tube with a tape-on label that will stay on the tube at liquid nitrogen temperatures and using a marking pen with permanent ink that does not bleed if exposed to acetonitrile. Mince gel band into 1 mm^3 pieces.

2. Equilibrate gel pieces in 0.5 mL 100 mM ammonium bicarbonate buffer (0.395 g/50 mL) at room temperature for 15 minutes with gentle agitation on vortex mixer. This step may be optional.

3. Wash gel pieces with 500 µL wash solution (50 mM ammonium bicarbonate/50% acetonitrile) 15 min, with gentle agitation (on Vortexer at the lowest setting). Discard wash solution. Repeat wash at least two times more. Most of the stain should have been removed from the gel pieces.

4. Rinse gel pieces briefly with 500 µL 100% acetonitrile and discard the rinse solution. Dehydrate the gel pieces with 500 µL 100% acetonitrile for 20 minutes at room temperature with gentle agitation. Discard acetonitrile, and allow gel pieces to air-dry.

5. Reduce the in-gel protein with dithiothreitol (150 µL 10 mM DTT in 100 mM ammonium bicarbonate; 1.542 mg/mL) for 30 minutes at 56° C.

6. Cool the sample to room temperature, remove and discard DTT solution.

7. Alkylate the in-gel protein with iodoacetamide (100 µL 50 mM IAA in 100 mM ammonium bicarbonate; 9.25 mg/mL) **in the dark** at room temperature for 30 minutes. Remove IAA solution. **Be sure to wear gloves while handling IAA. When finished with IAA solutions, neutralize them with a two-fold molar excess of DTT. Discard the neutralized IAA.**

8. Wash the gel pieces 1 time at room temperature for 15 minutes with 500 µL of the wash solution (50 mM ammonium bicarbonate/50% acetonitrile).

9. Rinse gel pieces briefly with 500 µL 100% acetonitrile and discard. Dehydrate the gel pieces for 20 minutes at room temperature with 500 µL of 100 % acetonitrile. Discard acetonitrile.

10. Rehydrate gel pieces for 2 hours at 4°C (on ice) in 30 µL of 0.02 µg/µL trypsin (Promega, modified porcine, TPCK-treated, Cat#: V5111) in 40mM ammonium bicarbonate (aq), 10% acetonitrile, in water [5]. Be sure that enough volume is added to ensure complete rehydration of gel pieces. More than 30 µL trypsin solution may be needed to completely rehydrate pieces of gel from a large band. Promega trypsin is sold

as 20 µg dried protein/vial. Reconstitute the trypsin at 1 µg/µL in the 50 mM acetic acid solution shipped along with the enzyme. Freeze this solution at -80°C in aliquots. Thaw and dilute the required amount of stock solution in the digestion buffer just before needed. Note that Promega Mass Spec grade trypsin may be used. Other sources of trypsin (for example, Sigma's mass spec grade trypsin) have not been tested recently by the Proteomics Lab, but some of them may perform satisfactorily. Avoid repeated freeze/thaw cycles of stock trypsin solution.

11. After two hours, replace the trypsin solution with 30-50 µL 40mM ammonium bicarbonate (aq), 10% acetonitrile, in water [5]. Place the digestion tube in a tube rack and wrap the rack plus tube completely with aluminum foil. Incubate at 37° C for 16-18 hours (overnight). The foil wrap helps minimize the amount of condensate that collects inside the reaction tube cap during the incubation and, thus, prevents the gel pieces from drying out overnight.

12. Remove the digest tube from the incubator and centrifuge briefly to deposit all liquid in the bottom of the tubes. Centrifugation in a benchtop minifuge for 15 seconds is adequate. Transfer supernatant = digest solution (containing tryptic peptides) to a clean 0.5-mL **natural (not colored) Eppendorf Safelock microcentrifuge tube** with a small hole poked into the cap. (Use a 22 gauge disposable syringe needle to poke the hole in the cap. The hole is so the tube can be kept capped during the final lyophilization step to be done in the Proteomics Center.) Add 8 µL of extraction solution (see below) to the tube per 30 µL of digest solution. This is done to acidify the digest and, thus, inactivate the trypsin. Keep the acidified digest solution on ice.

13. Add 25-50 µL of extraction solution (60% acetonitrile, 1% TFA) to gel pieces and agitate gently by vortexing at lowest setting. Extraction solution is made by combining 600 µL 100% ACN, 300 µL fresh HPLC-grade H_2O, and 100 µL of a fresh 10% TFA (aq) stock solution. Use the smallest volume of extraction solution possible to minimize dilution of the peptides. Extract for at least 10 minutes.

14. Spin down sample by brief centrifugation (just a quick spin in a minifuge or microfuge).

15. Pool the extract (containing additional tryptic peptides) with the digest solution on ice (step 12 above).

16. Extract the gels again with an additional 25-50 µL of extraction solution. Spin down sample and transfer the extract to the digest solution tube from step 12.

17. Freeze the pooled digest and gel extraction solutions using liquid nitrogen. Store at -80°C. Store the digested gel pieces at -80°C. A repeat of the digestion may be done if one discovers that the trypsin digestion failed for a band that was known to contain sufficient protein (on the basis of good staining intensity).

Notes

- Always use HPLC-grade water (MilliQ 18 mega ohm x cm), HPLC-grade solvents, and sequencing grade TFA.
- Prepare ammonium bicarbonate buffer and all reagent solutions on the day that they are to be used. Prepare DTT, IAA, and diluted trypsin solutions just before addition to the samples.
- Gel staining and preparation of peptides must be performed with lab ware that has never been in contact with nonfat milk, BSA, or any other protein blocking agent to prevent carryover contamination.
- Always use powder-free gloves when handling samples. Keratin and latex proteins are potential sources of contamination.
- Never re-use any solutions, abundant proteins will partially leach out and contaminate subsequent samples.

References

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