In-Gel Digestion Protocol for LC-MS

Reagents
Isopropanol (HPLC grade, EMD UN1219).
Acetonitrile (HPLC grade EMD OmiSolv AX0142-1)
Ammonium Bicarbonate (AB) (Fischer Scientific A643-500)
Formic Acid (Suprapur, EMD UN1179)
Trypsin (Promega)
Dithiothreitol and iodoacetamide

Solutions and buffers
Destaining solution: 50% acetonitrile / 200mM AB (pH 8) (0.8 g in 50 mL). Prepared fresh weekly.
25mM AB (pH 8) (0.1g in 50mL) Prepared fresh weekly.
Trypsin solution: Freshly prepared 10ng/µL trypsin solution in 25mM AB (pH 8)
LC-MS mobile phase A: 0.1% formic acid. Prepared fresh weekly
If reduction desired: *Reducing solution (8µL of 1.2 M dithiothreitol in 992 µL 25 mM AB) and Alkylating solution (18 mg iodoacetamide in 1.0 mL 25mM AB)*

Procedure
All the processes up to overnight digestion should be done in a laminar hood or a clean part of the lab.

1. Excision of band: Cut out the band of interest very precisely and divide into 1-3 mm pieces. Transfer them into a 0.65mL microfuge tube pre-washed with 50% isopropanol or the destaining soltuion

2. Destaining: Add 100 µL of the destaining solution and leave it for 10 min. Remove and discard the solution. Repeat the procedure at least 2 more times. (All the stain is washed. If not, you need more destaining, overnight in extreme situations for reall dark bands)

3. Dehydration: Add 100 µL of acetonitrile and discard it right away. Add 50 µL of acetonitrile and wait for 10 minutes to complete the dehydration. Remove acetonitrile and leave the gel pieces to air dry in the laminar hood for 30 min.

3a Reduction and alkylation (OPTIONAL): *If reduction and alkylation are desired, add 100 µL of reducing solution to the dried gel and leave it at room temperature for 30 min. Remove the reducing solution and add 100 µL of alkylating solution. Leave it at RT for 30 minutes in the DARK. Remove the alkylating solution and add 50 µL acetonitrile to cover the gel pieces. Allow 10 minutes to dehydrate the gel pieces again. Remove acetonitrile and let it air dry in laminar hood for 30 minutes.*

4. Digestion: To the dried gel pieces, add 10 µL trypsin solution and 25mM AB to cover the gel pieces. Place the tube in 37 ^{o}C incubator for 30 min. Make sure gel pieces are completely immersed in buffer solution and use 25 mM AB to cover them. Leave the microfuge tubes at 37 ^{o}C for 12-20 hr.

8. Peptide extraction: Remove the tubes from incubator and spin them for 30 sec. Using a gel loading pipette tip, transfer the solution to an autosampler vial insert. Add 50 µL of 50% acetonitrile 5% formic acid solution to the microfuge tube with gel pieces and sonicate it for 15 min. Transfer the solution to the same autosampler insert to combine with the first extract. Place the insert into an eppendorf tube and SpeedVac it until 5-10 µL is left (try 10 min with medium heating first). Complete the solution in inserts to 20 µL with the LC-MS mobile phase A while making sure there is air space left at the bottom. Place them in automsampler vials store the vials at 4 ^{o}C if they will be analyzed within a day or so otherwise freeze them

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