In-gel Digestion of Excised Protein Spots (Silver Stained Gels) with Destaining

1. Washing/Destaining
    a. Wash gel twice with dd(doubly deionized) H2O for 15 mins
    b. Excise bands/spots (cut as close to the band/spot as possible to minimize excess Polyacrylamide gel material), and cut into1 mm cubes and place them in an Eppendorf tube (Polypropylene tube only-- Polypropylene Eppendorf tube, Sarstadt 72.690 & 72.699 (1-800-257-5101)).
    c. Dry samples in a speed vacuum at around 56~60°C for about 45 min, the gel pieces must shrink and be completely dehydrated.
    d. Add 40 µl of 1:1 solution of 30 mM Potassium Ferricyanide and 100 mM Sodium Thiosulfate, incubate at room temperature for 15 min.
    e. Pull off solution and discard, add 40 µl of 100 mM Ambicand incubate at room temperature for 15 min.
    f. Repeat 1e) 3 times
    g. Pull off solution and discard, dry the samples in a speed vacuum as in 1c)
2. Reduction/Alkylation
    a. Remove samples from the speed vacuum and let cool.
    b. Add 40 µl of 10 mM DTT/100 mM Ambic and incubate at 56°C in a water bath or thermocycler for 45 min.
    c. Remove samples and let cool.
    d. Pull off solution and discard, immediately add 40 µl of 55 mM IAA/100 mM Ambic, then incubate at room temperature for 30 min in the dark.
    e. Pull off solution and wash with 40 µl of 100 mM Ambic, then incubate at room temperature for 5 min.
    f. Add 40 µl of acetonitrile to make 1:1 solution of Ambic and ACN, then incubate at room temperature for 15 min.
    g. Pull off solution and discard, dry gel pieces in speed vacuum as in 1c).

3. Digestion/Extraction

a. Add 40 µl or enough trypsin solution to cover gel pieces and incubate at 4°C for 45 mins (use ice bath or thermocycler, add more solution if pieces absorb all the liquid).
b. Pull off excess solution and discard, add 40 µl (or enough to cover gel pieces) same buffer but without trypsin and incubate at 37°C for 16 hrs (overnight).
c. Pull off supernatant and save at 3°C.
d. To gel add 20 µl of 25 mM Ambic and incubate at room temperature for 15 min.
e. Add 20 µl acetonitrile to make 1:1 solution of Ambic/ACN and incubate for 15 min.
f. Pull off the supernatant and combine it with the one from c).
g. To gel pieces add 20 µl of 5% Formic Acid, then incubate at room temperature for 15 min.
h. Add 20 µl acetonitrile to make 1:1 solution of ACN/Formic Acid, then incubate for 15 min.
i. Repeat 3f), 3g), 3h), 3f).
j. To pooled supernatant add 10 mM DTT to give final concentration of 1 mM DTT .
k. Completely dry the supernatant, which is the digestion extracts, in speed vacuum.
l. Resuspend the digestion extracts in 15 µl~20 µl of 5% Formic Acid for MS or MS/MS analysis.

Note:

- Larger spots/bands may require more solution. Please adjust the volume accordingly.
- For MALDI analysis, Ziptips(pipette tips with RP-C18 at the tip) should be used to clean up the in-gel digest samples to get rid of salts and detergents prior to spotting the sample on the MALDI state. Routine sensitivities on realworld samples are in the 10~50 fmol rage. Single digit femto-mole level can be reached occasionally. For a routine ziptip protocol, see here.

Some background information:

Mann et. al. have published a paper in Anal Chemistry (1996 vol.68 pg.850-858), where they provide a alternate silver staining method that is MS compatible. However, this staining method is prone to high background, describe here a Rabilloud silver staining method that is better and is also mass spec compatible. Recently Gharahdaghi et al. published a paper in Electrophoresis (1999 vol.20 pg.601-605) that demonstrates a method which increases sensitivity after destaining (remove of silver ions) of the gels pieces prior to enzymatic digestion. So the protocol for enzymatic digestion from silver stained spots is a modified version of Mann's protocol. This protocol has been used with routine real world protein sample analysis from gels in the 10~50ng and higher range.