In-solution trypsin digestion

1. PRINCIPLE

In many proteome studies sample analysis is performed directly from the liquid phase. Trypsin is used here as the endoprotease, but in practice others are used as well. The choice of enzyme will be project and sample dependent. Trypsin is particularly useful as it generates usually many peptides of ideal length for analysis. It also cleaves C-terminal to lysine and arginine amino acids, resulting in a N-terminal amine group that can accept a proton and a basic side chain residue of the K/R that will also take up a proton.

2. MATERIALS

Water, MilliQ
Trizma base, Sigma T1503
HCl(1 – 5 M) for pH adjustment
1,4-dithiotreitol (DTT) (purity >99%), Ducheta D 1309
Iodoacetamide (IAA) (purity >99%), Sigma I-6125
Sequencing Grade Modified Trypsin, Promega V5111
Urea (purity >99%)

3. SOLUTIONS

1 M Tris-HCl stock solution, pH 8.0
Weight accurately 6,057 g of Trizma base in a 50 ml Falcon tube and dissolve it with water. Adjust the pH to 8.0 by HCl. Fill to 50 ml mark with water. This 1 M stock solution can be stored for six months at 4 °C.

50 mM Tris-HCl, 10 ml
Dilute from 1 M stock solution by mixing 500 µl of Tris stock solution with 9500 µl water. Note! Tris-HCl buffering capacity is temperature dependent and the solution should be used at RT.

6M Urea
Dissolve 1.0 g of urea in 2.5 ml of 50 mM Tris-HCl solution.

Reducing reagent
Dissolve 30 mg of DTT in 1.0 ml of 50 mM Tris-HCl solution to make 200 mM DTT.

Alkylating reagent
Dissolve 36 mg (0.036 g) in 1.0 ml of 50 mM Tris-HCl solution to make 200 mM iodoacetamide.

Trypsin solution
Dissolve 20 µg of trypsin (one vial) in 20 µl of buffer provided with Promega's trypsin. This 1.0 µg/µl solution can be used according the protein content in the sample.

4. SAMPLE CLEAN UP USING ACETONE PRECIPITATION

Add 4-5 volumes of cold acetone (-20 ºC) to the sample.
Keep at -20 ºC for at least 2 hours or o/n. Centrifuge for 10 min.
Remove the supernatant and wash the pellet with chilled acetone. Repeat centrifugation and remove all acetone. Air dry the pellet for 5 min. Do not over dry the pellet or it will be difficult to resuspend. Resuspend the pellet in freshly prepared 6.0 M urea, 50 mM Tris-HCl.

5. PROTEIN CONCENTRATION DETERMINATION

It is important to know how much protein is in the sample, so that one can add an optimum amount of trypsin. Use Bradford assay to measure the protein concentration.

6. IN-SOLUTION DIGESTION

All reagents should be prepared immediately prior to use. The water used in all components of the procedure is MilliQ-type water. The urea, Tris-HCl, and other chemical should be of the highest grade available. Trypsin (or other enzyme) should be sequencing-grade and ideally be treated to generate a minimum autolysis products.

Day One:

1. Reconstitute sample in approximately 100 µl of 6.0 M urea in a 1.5 mL plastic centrifuge tube.
2. Add 5 µl of Reducing Reagent and mix the sample by gentle vortex.
3. Reduce the mixture for 1 hour at room temperature or in an oven at 37 ºC.
4. Add 20 µl of Alkylating Reagent and alkylate for 1 hour at room temperature in the dark (you can use aluminum foil to cover up the sample).
5. Add 20 µl of Reducing Reagent to consume any leftover alkylating agent (so the trypsin is not alkylated).
6. Add 900 µl of 50 mM Tris-HCl solution to dilute the urea before digesting it with trypsin.
7. Add trypsin in appropriate ratio (1:30) to approximate amount of protein by weight. Digest over night at 37 °C.

Day Two:

To reduce the urea concentration before LC-MS/MS analysis, samples should be either diluted or urea should be removed by desalting with C_{18} ZipTips, OMICS tips or similar products.