In-solution protein digestion

Proteins in solution are usually denatured by boiling or using denaturing buffers. During this step, the disulfide bonds must be reduced, and the sulfhydryl groups must be alkylated to prevent the disulfides from re-forming. The protein samples are then incubated with trypsin for several hours, and the resulting peptides can be analyzed by MS.

Denaturing buffers contain chaotropic agents, salts, and detergents at concentrations that inactivate trypsin. Before adding trypsin, you should desalt your protein sample and remove detergents. There are a number of the detergent removal and desalting options: detergent removal spin columns, size-exclusion and MW cut-off spin columns, ion-exchange membranes and resins, etc.

Reagents

Digestion buffer: 4 mg/ml ammonium bicarbonate in water (50 mM pH 8.5).

Reducing reagent: 15 mg/ml DTT (100 mM, Sigma D0632) in digestion buffer.

Alkylating reagent: 18 mg/ml iodoacetamide (100 mM, Sigma I1149) prepared fresh in digestion buffer.

50 mM acetic acid: Add 15 µl glacial acetic acid to 5 ml H_2O.

Proteomics grade trypsin (e.g. Sigma T6567-5x20UG or Thermo Pierce 90057, 5 vials × 20 µg lyophilized powder). Trypsin, 20 µg can be dissolved in 20 µl of 1 mM HCl or 50 mM acetic acid, pH ~ 3, aliquoted and stored at -20 °C (stock solution).

To prepare activated (or working) trypsin solution, dilute trypsin stock solution with digestion buffer 10-fold to 0.1 µg/µl concentration.

Procedure

1. Volumes are approximate, it is a sample preparation procedure after all. Trypsin should not exceed 5% of the total protein, provided the protein concentration range is known.
2. Dilute 1-10 µg protein in 100 µl digestion buffer, add 2 µl of DTT to make final concentration 2 mM, incubate at 60 °C for 30 minutes.
3. Add 8 µl IAA to make final concentration about 10 mM, incubate in darkness for 30 minutes (make sure there is 2-4 times more IAA than DTT to react excess DTT).
4. Wait for the mixture solution to cool down, add 8 µl DTT (2 mM + 8 mM = 10 mM) to neutralize IAA and stop the reaction.
5. Depending on the protein amount, add trypsin from stock solution (0.1 µg/µl) in 50:1 ratio (50 parts protein to 1 part trypsin). Incubate at 37 °C overnight (shaking at 300 rpm).
6. Store digested peptides at -20 °C for LC-MS analysis. Dilute the sample to inject 50-200 fmol.

If protein sample contains detergents, salts, or chaotropic agents, perform buffer exchange after alkylation using a 3,000 MWCO centrifugal filter. It will be impossible to remove detergents after the digestion; and most detergents are not compatible with LC-MS analysis.

Minimum sample amount required for MS analysis is in the fmol/µl range (ng/µl). Solutions of peptides at very low concentrations (e.g. less than 100 fmol/µl) should not be stored for more than 1-2 days.