Determination of ubiquitylation sites

(Cotter Lab, 2004)

Tryptic digestion of ubiquitylated proteins results in peptides containing this PTM site with GlyGly-modified lysine residues. Prior N-terminal sulfonation improves their location in the sequence by enabling de-novo sequencing, and also provides a set of signature neutral loss peaks that can be used to quickly identify peptides carrying the modification.

1. Carry out tryptic digestion of ubiquitylated proteins, or protein mixtures containing ubiquitylated proteins, using protocols for Bovine pancreas modified trypsin. If required, fractionate resultant peptide mixture using reversed-phase HPLC.

2. Carry out the sulfonation reaction for each peptide fraction in a 0.6 mL Eppendorf tube by mixing 9 µL of reagent solution: SPITC (10 mg/mL) in 20 mM $NaHCO_3$ with 1 µL of peptide solution (~10-100 pmol), as described above. After incubation for 30 min at 55^{o} C, terminate the reaction by adding 1 µL of 1% trifluoroacetic acid (TFA).

3. Analyze each fraction using tandem mass spectrometry. All peptide MS/MS spectra will show the loss of the sulfonate tag HO_3S-C_6H_4-NCS (loss of 215 Da) and a less abundant loss of the partial tag HO_3S-C_6H_4-NH_2 (loss of 173 Da).

4. Peptides containing the ubiquitylation site will, after tryptic digestion, carry a GlyGly-modified lysine residue. As this branch constitutes a second N-terminus, these peptides will carry two sulfonate tags. Thus, there will be the addition losses of 430, 388 and 346 Daltons, corresponding to the loss of two tags, one tag and one partial tag, and two partial tags, respectively. These additional losses provide a *signature* for peptides carrying ubiquitylation sites.

5. The remaining ions in the MS/MS spectrum can be attributed to y-series (peptide sequence) and y'-series (losses of G and GG) ions. The modification site K_{GG} appears in this ion sequence as a mass difference corresponding to two glycine and one lysine residue.