PP Proteomics SS2018

Extraction and trypsin in-solution digestion for leaves

Reagents (all reagents are freshly prepared and ready to use)

- Extraction buffer: 8 M Urea in 50 mM HEPES pH 7.8
- Precooled Aceton + 0.5% ß-Mercaptoethanol (ME)
- Digestion buffer: 50 mM NH_4HCO_3 (AmBic), 10% ACN, 20 mM $CaCl_2$
- Alkylation buffer: 1 mL stock (5 µg/µL iodoacetamide (IAA) in 100 mM AmBic pH 8)
- 200 mM Dithiotreitol (DTT) stock
- Bradford (ready to use)
- Trypsin beads (ready to use)
- MS sample buffer: 0.1% FA in 2% ACN

Method

Extraction

1. Labelling and weighing of 2 mL Eppi`s - note down!
2. Harvest samples (2 leaves per plant), transfer into the 2 mL Eppi and freeze immediately in liquid N
3. Weight Eppis with samples (FW) – note down!
4. Grind Samples in Retsch Mill
5. Add 0.5 mL **extraction buffer** and homogenize
6. Centrifuge at 10,000 x g, 10 min, 4°C
7. Transfer the supernatant to 15 mL tube, mix thouroughly and aliquot to two replicates in 15 ml tubes

Precipitation

8. Precipitate supernatant in ice-cold acteton,0.5% mercaptoethanol (5 x urea buffer volume), -20°C, over night
9. Vortex shortly
10. Centrifuge at 4000 g (max speed), 4°C, 15 min

11. Discard the supernatant

12. Wash the pellet once with 2 ml ice-cold 100% methanol
13. Centrifuge at 4000 g, 4°C, 10 min
14. Wash with 2 ml ice-cold 100% acetone
15. Let Falcon tubes dry under the hood for ~5-10 min
16. Dissolve precipitated protein in 700µl urea buffer

Bradford analysis: Calculation of Protein concentration

The protein concentration will be a photometric analysis at 595nm in 96 well-plate.

1. Pipette 2 µL of sample to 18 µL H_2O (three replicates per sample)
2. Add 200 µL Bradford reagent to each sample well
3. Measure well plate after 5-10 minutes in a photometer at 595 nm.
4. Use BSA Calibration Curve for protein concentration calculation
5. **Prepare two fractions; one for In-solution and one for In-gel digestion (Figure 1). In gel digestion see SDS-PAGE protocol**

<u>Digestion</u>

1. Transfer a sample volume corresponding to 50 µg to a 2 mL safelock Eppi
2. Reduction: add DTT to final concentration of 5 mM (Stock 200 mM)
3. Incubate at 37 °C 700 rpm for 30 min
4. Add **alkylation buffer** (1 µL per 50 µg of 1M stock) and incubate 45 min at RT in DARK
5. Nutralize remaining IAA by adding **20 µL DTT stock**, vortex, incubate 30 min at RT in DARK
6. Add trypsin buffer (3 times the sample volume) and trypsin (2 µl per 50 µg protein)
7. Run digestion for over night at 30°C in an rotating incubator.

Table 1: Digestion buffer (prepare fresh 1 M stock: AmBic (ammonium bicarbonate (NH4HCO3) (79.06 M)), add AmBic and DTT just before use

Trypsin Buffer	stock	5 ml	10 ml
H_2O		4.2	8.4
ACN (10%)	100%	0.5	1
AmBic (50 mM)	1 M	0.25	0.5
CaCl (2 mM)	200 mM	0.05	0.1
Dithiothreitol (5 mM)	200 mM	0.125	0.25

Peptide desalting

For desalting, digests are added to a SPEC18 System as follows (always 100µl):

1. Wash C18 columns with 5% ACN, 0.1% FA
2. Equilibrate with water (0.1% FA) twice
3. Add sample –dispense and aspirtate sample 5-10 times →Vacuum
4. Wash with water (0.1% FA) twice →Vacuum

   Elute peptides:
5. Exchange collecting tray!
6. 70% ACN (0.1% FA) - twice →Vacuum
7. Transfer flowthrough to siliconized tube (0.5 mL Eppendorf low binding).
8. Dry in speed vac

   Samples can be stored at –20°C until MS analysis.

   Prior to MS analysis: desolve peptide pellet in **MS sample buffer (0.5µg/5µl).**

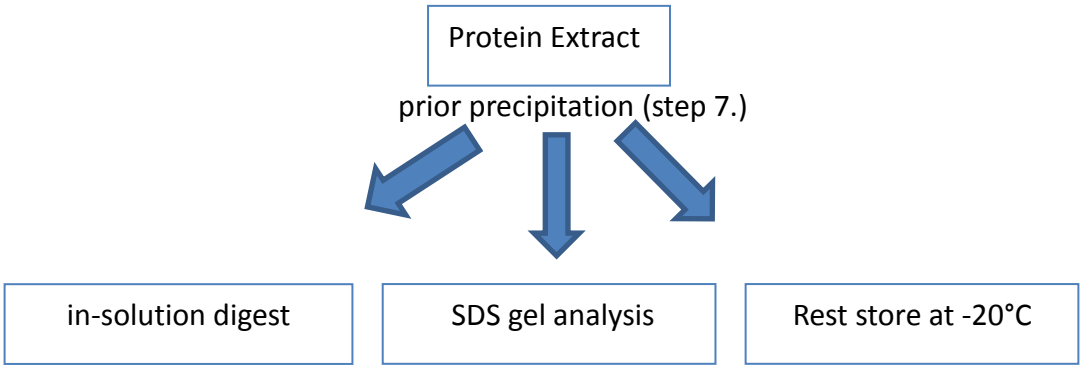

Figure 1 After Bradford Analysis, split sample(s) in 2 Fractions: a) 50 µg for in-solution digestion b) 10 µg for gel-analysis; store rest at -20°C.