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In gel protein digestion using trypsin as protease, and extraction of liberated peptides

In gel digestion (day 1)

Silver removal (only silver stained gels);

Cut gel bands/spots into 1 mm cubes in eppendorf tubes. Wash once with 500 µl water (incubate at room temperature (RT) for 20 min. in an 1.5 ml Eppendorf Thermomixer comfort, Eppendorf AG, Germany). Add 200 µl destain solution *(see right panel)*, and incubate at RT in Eppendorf mixer for 5 min (slow agitation).

Remove supernatant, and wash gel pieces by adding 100 µl MilliQ water. Incubate at RT in Eppendorf mixer for 5 min (slow agitation), and discard supernatant. Repeat 4 times.

Destain solution:

Mix equal volumes of 30 mM potassium ferricyanide (10 mg $K_3Fe(CN)_6$/ml MilliQ water) and 100 mM sodium thiosulfate (16 mg $Na_2S_2O_3$/ml MilliQ).

Gel washing;

Cut gel bands/spots into 1mm cubes in eppendorf tubes. Add 50-100 µl wash solution *(see right panel)* to each sample, and incubate at RT for 20 min. in the Eppendorf mixer. Remove supernatant, and repeat wash once.

Discard supernatant, dry gel pieces preferably in vacuum, a "Rotavapor" *(or by adding 50 µl ACN and shake for 2 min. Remove ACN. The gel pieces should now be white and sticky).*

Wash solution:

Add 250 µl 1M Ambic (frozen stock solution of 1M ammoniumbicarbonate in MilliQ water, 80 mg/ml) to 4750 µl MilliQ water and 5 ml ACN (acetonitrile, HPLC grade)

In gel reduction og alkylation (Cys);

Reduce cysteins by adding 50 µl 10 mM DTT (DiThioTreitol from Amersham Biosciences, #171318-02) to the dried gel pieces *(see right panel)*, and incubate at 56 ºC for 45 minutes.

Cool samples, and remove DTT solution. Immediately add 50 µl 55 mM IAA (iodoacetamide, Sigma Aldrich, I-6125) for cystein alkylation *(see right panel)*, and incubate in the dark at room temperature for 30 min. Remove IAA solution, wash twice as described above, and dry gel pieces in vacuum, a "Rotavapor".

10 mM DTT in 100 mM Ambic:

Add 10 µl 1M DTT (Frozen stock solution of 154 mg DTT/ml MilliQ water) to 890 µl MilliQ water and 100 µl 1M Ambic.

55 mM IAA in 100 mM Ambic:

Add 10 mg IAA to 900 µl MilliQ water and 100 µl 1M Ambic.

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In gel protein digestion;

Coomassie spots: Add 20 - 40µl 6ng/µl Trypsin Porcine (from Promega, #V 511A) to each sample *(see right panel)*, and rehydrate on ice for 30 min.

Silver and Sypro spots: Add 20 - 40µl 3ng/µl Trypsin Porsine (from Promega, #V 511A) to each sample *(see right panel, but using 5µl Trypsin stock solution)*, and rehydrate on ice for 30 min.

Incubate samples for 16 hours at 37 °C in a hot cabinet.

Digestion buffer:

Add 50 µl 1M Ambic, 10µl 0.1M $CaCl_2$ and 50 µl ACN, to 890 µl MilliQ water.

6ng/µl Trypsin:

Mix 10 µl Trypsin Promega Porsine stock solution (100ng/µl dissolved in 50 mM acetic acid) and 160 µl digestion buffer (see above).

Peptide extraction (day 2)

Extraction of peptides;

Cool and spin samples. Pull off and save supernatant in **new eppendorf tube**.

Add 30-50 µl 5% FA (formic acid), and incubate at room temperature for 20 min in the eppendorf mixer. Pull off supernatant and pool with the first extraction.

Add 30-50 µl 60% ACN/0.1 % FA to gel samples, and incubate for 20 min. in the eppendorf mixer. Pull off supernatant and pool with the two former extractions.

Vacuum dry solution in a Rotavapor (Concentrator 5301 from Eppendorf AG, Hamburg, Germany) till it remains 10-15 µl samples. If necessary, add 15 µl 0.1% TFA and vacuum dry to about the same volume as above (samples should not contain ACN).