Alkylation and In-gel Trypsin Digestion

1. Protocol usage

To identify proteins which have been separated by gel electrophoresis.

2. Principle

Gel separated proteins are proteolytically digested into peptides prior to identification by mass spectrometry. The process involves 'in-gel' reduction and alkylation, followed by digestion of the proteins and extraction of the peptide from the gel piece(s).

3. Protocol workflow

A protein band or spot is cut from a gel. The gel piece is washed to remove impurities, such as e.g. SDS. Any disulfide bound cysteines in proteins are reduced and reformation of disulphide bridges is prevented by alkylation. Proteins are digested to peptides by trypsin and the peptides are extracted from the gel:

- Preparing the gel piece
- Reduction
- Alkylation
- Digestion
- Extraction of peptides

4. Reagents and equipment

H_2O, MilliQ
Acetonitrile (**ACN**) (purity >99,8%)
NH_4HCO_3 (purity >99%)
1,4-dithiotreitol (**DTT**) (purity >99%)
Iodoacetamide (**IAA**) (purity >99%), Sigma I-6125
Sequencing Grade Modified Trypsin, Promega V5111
Formic Acid (**HCOOH**) (purity 98-100%)
Scalpel
Tweezers
Eppendorf tubes 1,5 ml or 0,5 ml (To avoid plasticizer contamination, tubes made of high quality polypropylene must be used.)
Tips
Gloves
Heating block
Vacuum centrifuge (SpeedVac)

5. Solutions

1 M ammoniun bicarbonate - NH_4HCO_3 stock solution

Weight accurately 15,81 g of NH_4HCO_3 in a 200 ml flask and fill to mark with water. Filter through 0,45 μM filter. The pH of the solution should be 8-9. This 1 M stock solution can be stored for one month at 4 °C. For 100 mM solution, mix 1,0 ml stock solution and 9,0 ml MilliQ water.

20 mM DTT

Accurately weigh DTT in an Eppendorf tube. Dissolve in 100 mM NH_4HCO_3 in a ratio of 3,08 mg DTT **:** 1 ml NH_4HCO_3. <u>This solution should be prepared fresh.</u>

55 mM IAA

Accurately weigh IAA in an Eppendorf tube. Dissolve in 100 mM NH_4HCO_3 in a ratio of 10,2 mg IAA **:** 1 ml NH_4HCO_3. T<u>his solution should be prepared fresh and kept in the dark* at RT.</u>* cover with foil or store in cupboard

0,02 µg/µl trypsin solution

<u>Note:</u> When working with trypsin, keep it always on ice. The trypsin stock solution is prepared by dissolving 20 µg aliquot of trypsin in 20 µl of Promega trypsin dilution buffer. The stock solution can be stored up to one month at -20°C. Directly prior to use, dilute the trypsin stock solution (1 mg/ml) in a ratio 1:50 with a solution containing 40 mM NH_4HCO_3 and 10 % ACN.

6. Instructions

When you prepare solutions and handle your samples keep in mind that most common keratin contaminations arise from dust, hair or skin.

The volumes here are calculated for thin slices from a 1-DE gel band, so with a bigger piece of gel or small gel spot the volumes should be adjusted.

A. PREPARATION OF THE GEL PIECE

Excise the stained protein band from the gel with a clean scalpel. If the gel is dried, rehydrate the gel piece in water and remove the cellophane. Cut the gel into approx. 1x1 mm pieces in an Eppendorf tube. Wash the gel pieces thoroughly by 3 x 10 min washes in H_2O.

Coomassie blue stained proteins

Wash Coomassie stained gel pieces twice with 200 µl of solution containing 0,04 M NH_4HCO_3/ 50 % ACN for 15 min at 37 °C or as long as required to remove all blue colour. Shrink the gel pieces by adding at least 200 µl 100% ACN. Wait until the gel pieces become white (about 5-10 min) and remove all ACN.

For **silver stained proteins**, no destaining is needed. Shrink the gel pieces by adding at least 200 µl 100% ACN. Wait until the gel pieces become white (about 5-10 min) and remove all ACN.

B. REDUCTION

Rehydrate the gel pieces in 100 µl 20 mM DTT for 30 min at 56°C. Remove the excess liquid and shrink the gel pieces again with ACN as above.

C. ALKYLATION

Rehydrate the gel pieces by adding 100 µl 55 mM IAA for 20 min in the dark RT. Remove the excess liquid and wash the gel pieces <u>twice</u> with 100 µl 100 mM NH_4HCO_3.

Dehydrate the gel pieces again with ACN as above, remove all ACN (and dry in a vacuum centrifuge for 5 min to evaporate all ACN).

D. DIGESTION

Add 10 µl 0,02 µg/µl trypsin solution and allow to absorb for 20 min on ice. Check if all solution was absorbed and add more if needed. Gel pieces should be completely saturated with trypsin solution. After 20 min, add about 20 µl solution containing 40 mM NH_4HCO_3/10 % ACN to completely cover the gel pieces. Incubate up to 18 hours at 37 °C (overnight).

E. EXTRACTION OF PEPTIDES

Add about 30-60 µl ACN (equal volume with the digestion mixture), vortex and incubate 15 min at 37 °C. Collect the supernatant to another eppendorf tube and repeat the extraction with 150 µl solution containing 50 % ACN / 5% HCOOH. Dry the solution in a vacuum centrifuge and store the dried peptides at -20 °C. Immediately prior to MS analysis, dissolve the peptides in 10 µl 2% HCOOH by vortexing, incubate at 37 °C for 15 min and vortex again.

7. QC control

Sometimes we have problems with contaminating proteins such as keratins, or no proteins can be identified. To confirm that all reagents are working, or to define the origin of possible protein contaminations such as keratin, use a positive and a negative control for each batch of analyses.

A positive control is a gel piece containing 0,5 pmol BSA and negative control is a similar size gel piece without any protein. Customers using the facility should include a positive and negative gel piece.
A successful digestion results in the identification of numerous peptides from BSA in the positive control, whereas in the negative control only trypsin should be identified.

8. References

Shevchenko et al. (1996) Anal. Chem. 68, 850-858.
Shevchenko et al. (2006) Nature Protocols 1(6), 2856-2860.