

## In-solution Protein Digestion with UREA Denaturing Agent and Trypsin Protease

Urea is a chaotropic agent and disrupts the three dimensional structure of proteins promoting denaturation. **However, urea + heat + protein = carbamylation;** urea in solution is in equilibrium with ammonium cyanate, that may decompose to ammonia and isocyanic acid (HNCO). Isocyanic acid attach the N-terminal of the protein, but also the side chains of lysine and arginine residues rendering a protein unsuitable for many enzymatic digests ( $\text{HN}=\text{C}=\text{O} + \text{H}_2\text{N}\sim \rightarrow \text{H}_2\text{N}-\text{CO}-\text{NH}\sim$ ). Urea will always degrade to isocyanic acid, so urea solutions must be made fresh, and it is recommended to add 20mM methylamine ( $\text{CH}_3\text{NH}_2$ ) to the urea solution prior to use (urea can also be removed before digestion using reversed phase chromatography)

### Protein solvation/denaturation (applies for 100 µg protein or lower)

#### Dissolving the protein pellet;

The pellet may be difficult to dissolve. Add **20µl urea solution** (see right panel) and pipette gently up and down, sonicate if necessary.

Add **20µl trypsin buffer** (see right panel), and incubate at RT in Eppendorf mixer for 5 min (slow agitation).

Urea solution; 8M Urea/20mM methylamine:

Add **480 mg Urea** (art. no. U1250, Sigma-Aldrich), **1.7µl 40 wt% methylamine in H<sub>2</sub>O** (art. no. 426466, Sigma-Aldrich) and **630µl dH<sub>2</sub>O**.

Trypsin buffer; 50mM Tris/1mM CaCl<sub>2</sub>:

Add **0.61g Tris** (art. no. 252859, Sigma-Aldrich) and **15mg CaCl<sub>2</sub> x 2H<sub>2</sub>O** (art. no. 21097, Sigma-Aldrich, stabilize trypsin) to about 90ml dH<sub>2</sub>O. Correct the pH to 7.8-8 with HCl and adjust the volume to 100ml. Store the solution at 4 °C.

## Reduction and alkylation

### Reduction:

Add **4µl 100 mM DTT** (*see right panel*), and incubate for 1 hour at room temperature (RT).

### Alkylation:

Add **5µl 200 mM IAA** (*see right panel*) for cysteine alkylation, and incubate for 1 h at RT (dark).

To avoid unwanted protease alkylation, add **0.8µl 100 mM DTT**, and incubate 10 min. at room temperature.

### 100 mM DTT in MilliQ water:

#### **Add 15.4 mg DTT**

(DiThioThreitol, art. no. D-9163, Sigma-Aldrich) to 1ml dH<sub>2</sub>O (may be aliquoted as a 1M solution, and kept in freezer).

### 200 mM IAA in MilliQ water:

#### **Add 18.5mg IAA**

(Iodoacetamide, art. no. I-6125, Sigma Aldrich) to 0.5ml dH<sub>2</sub>O (must be freshly made and kept in the dark).

## Digestion

### Sample dilution:

Add **110.2µl Trypsin buffer** (the urea concentration is now 1M).

### Trypsin:

Add trypsin at a concentration about 50 times lower than the amount of protein in the sample. If the sample contains approx. 100 µg protein, add 2µg of protease (*see right panel*). Measure pH using an indicator paper (litmus paper or similar), and incubate samples at 37°C overnight on a shaker

### 2µg Trypsin Porcine (4µl)

(Promega, art. no. V 5111):

Dissolve each ampoule (20 µg trypsin porcine) in 40 µl 50 mM acetic acid (resuspension buffer supplied from Promega with the trypsin powder). The trypsin concentration in this stock solution is then 0.5 µg/µl

## Acidification

In this final step, acidify with 10% TFA (trifluoroacetic acid) to a 1% final concentration of TFA. Proceed with desalting on OASIS C18, and dry in freezevac.