Digesting proteins in solution

General remarks
Proteins, unfortunately, show very individual behaviour upon digestion when performed in-solution. Accordingly, it is not easy to setup one standard protocol for all proteins. The protocol provided below is a guideline that has to be adapted to the properties of the individual protein(s) under investigation. Proteins can differ significantly in their solubility, charge, stability, as well as their susceptibility to various proteolytic enzymes.

Problem of insoluble proteins
Certain proteins, e.g. membrane proteins, are insoluble in aqueous solution without addition of detergents. As a further example, recombinant proteins purified from inclusion bodies are also insoluble in water. It may be necessary to use detergents in these cases (for example N-octylglycoside, dodecylmaltoside). However, as will be outlined later on, most detergents are incompatible with direct mass spectrometric analysis (for methods to remove detergents, refer to chapter “Reduction of disulfide bonds”).

Reduction of disulfide bonds
In most cases it will be necessary to reduce intramolecular disulfide linkages of the protein to be digested. This will help to unfold the protein chain and, thus, will increase the protein´s susceptibility for the proteolytic digest. Some proteins are completely resistant to proteolysis if they are not reduced in advance.
Proteins may have a very stable tertiary structure, so that efficient reduction can only be achieved under special denaturing conditions. To efficiently reduce e.g. antibodies, high concentrations of urea or guanidinium hydrochloride have to be applied. It is also possible to apply detergents such as SDS. All these approaches have at least one significant disadvantage: Usually, these additives used for denaturing are not compatible with the subsequent proteolytic digestion process (proteases are proteins themselves) and/or with the final mass spectrometric analysis. Typical protocols to address these problems are:

- Perform the reduction of the protein in 6M urea. Afterwards, dilute the sample to a final urea concentration of 1M for the digestion step.
- Use 1% SDS as a detergent for the reduction step, with subsequent dilution of the sample down to a SDS concentration of 0.1%.

Whenever detergents are used during the reduction step, addition of 1% n-octylglucopyranoside upon the addition of the enzyme will prevent the protease from being denatured. However, most detergents are incompatible with direct analysis by mass spectrometry and, unfortunately, are not easily removed from a sample solution.
In case of direct MALDI-TOF/TOF analysis, digested samples containing urea will require at least further dilution or even an additional cleanup step, using ziptip or similar devices, since the presence of urea in the sample may prevent the MALDI matrix preparation from crystallizing.
When using other detergents, methods like dialysis, ultrafiltration or precipitation of the protein using acetone or TCA may be considered to remove the detergent from the sample. In case of precipitation, subsequent reconstitution of the protein sample has to be performed in a protease compatible buffer. However, keep in mind that reduced proteins may be rather insoluble after precipitation, because their hydrophobic parts become exposed.

Proteolytic digest

Trypsin is the protease of choice in most cases. It is cheap and usually best suited for MS/MS experiments since the resulting peptides carry two positives charges at both termini. However, whenever trypsin fails to digest a protein at reasonable efficiency, alternative enzymes have to be considered, e.g. endoproteases Lys-C , Asp-N or Glu-C. When a protein needs to be characterized completely regarding its aminoacid sequence, multiple enzymes may be required to achieve 100% sequence coverage. If a protein turns out to be extremely resistant against proteases, chemical cleavage using cyanogen bromide followed by enzymatic digestion may be an option.

Addition of organic solvent to the digestion buffer may also help to increase the digestion rate. Trypsin will cleave in the presence of methanol up to a percentage of 30%. However, the occurrence of a higher frequency of unspecific cleavages under these conditions has been reported. Many proteases exhibit an increased cleaving activity at an acetonitrile percentage of 5 to 10%.

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Protocol for in-solution digestion using trypsin as an enzyme

(this is a standard protocol to be applied to proteins showing “normal” behaviour upon digestion; in case of more critical protein samples, refer to the advices mentioned above)

1) Dissolve the protein to be digested in a suitable digestion buffer, i.e. 50mM ammonium bicarbonate (ABC) in water.
2) To reduce disulfide bonds, add freshly prepared 45mM solution of dithiothreitol (DTT) dissolved in 50mM ABC in water. Per nmol of protein to be reduced, add 1µl of the DTT solution described above. Incubate for 15min at 50°C.
3) For alkylation of cysteins, add freshly prepared 100mM solution of iodoacetamide (IAA) dissolved in 50mM ABC. Per nmol of protein to be alkylated, add 1µl of the IAA solution dscribed above. Incubate for 15min at room temperature in the dark.
4) Destroy the excess IAA in the protein sample solution by adding another 1µl aliquote of the DTT solution used for reduction in step 2).
5) Add a small volume aliquote of trypsin stock solution to obtain a final enzyme : substrate ratio of 1:50 (mass:mass). Note: Make sure the added aliquot of the enzyme stock solution (which is normally stored at -80°C at acidic pH) will not drop the overall pH of the sample solution. This would affect the cleaving efficiency of the enzyme. After addition of the enzyme, incubate the sample solution overnight at 37°C.
6) After finished incubation, stop the enzyme activity by adding a small aliquote of highly concentrated (i.e. 10%) TFA.

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