LECTURE-9

Protein Digestion

HANDOUT

PREAMBLE

Proteomic study requires the separation of proteins and their identification. In most of the proteomic studies the proteins are separated by either electrophoretic method (1D, 2D-gel electrophoresis) or chromatography method like HPLC. In 1D and 2DE experiments the proteins are separated on the gel and protein spots of interest are excised from the gel and subjected to in-gel digestion. The extracted peptides are spotted on MALDI plate and identified by MALDI-TOF or subjected to LC-MS/MS. In case of in-solution digestion the protein samples are digested in solution itself and further separated using chromatography method and identified by using mass spectrometry. Proteins are digested into their corresponding peptides by using different proteases or chemical agents followed by the detection of peptides by using mass spectrometry. Identified peptide masses are matched with the database for the identification of the proteins.

OUTLINE

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1. Introduction

Invention of mass spectrometry revolutionized various fields of chemistry, biology and agriculture. Mass spectrometry is also having promising applications in various industries like food, pharmaceutical and petroleum industry. With the advances in soft ionization techniques and development of detectors for the mass spectrometer now it is possible to detect very small molecules to large molecules with great sensitivity. In chemistry mass spectrometer is used for the detection of the molecular mass of the synthesized chemicals and to check the purity of the sample. In biology generally it is use for the detection of the molecular mass of the protein/ peptides/ metabolites. Soft ionization methods are used for the detection of intact proteins' mass or protein identification.

2.Protein digestion

In the biological system most of the proteins have molecular mass more than 20 kDa. Those intact proteins can't be identified by using MALDI-TOF instrument. So prior to the detection, the protein should be cleaved into its corresponding peptide fragments using specific proteases. Trypsin is generally used for digesting the protein into peptide. Trypsin cleaves the peptide bond at the C-terminal of the lysine and arginine. It can't cleave the peptide bond if the lysine or arginine is followed by proline (Hustoft et al., 2012).

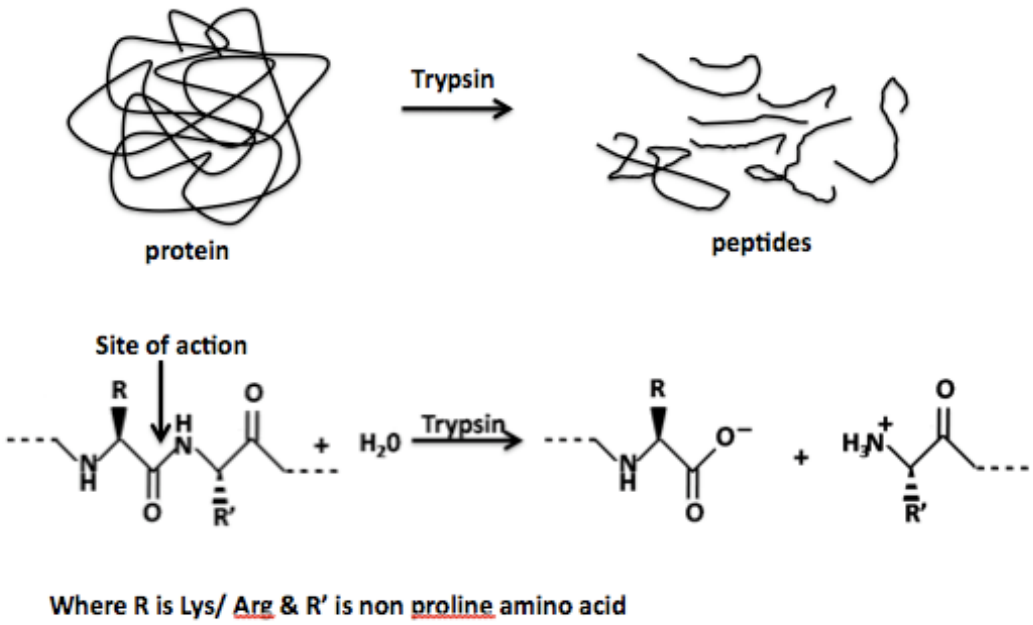

Fig.1. Process of enzymatic action by trypsin

Trypsin digestion of the proteins can be done in two different ways: In-gel digestion or In-solution digestion. If the proteins are first separated on a gel either by 1D or 2D- gel electrophoresis and further subjected to trypsin digestion, this method is called as "In-

gel digestion". If the protein extract is directly subjected to digestion in a buffer solution then this process is called as "in-solution digestion".

Trypsin is a serine protease, which digests the proteins into peptides. As the proteins in our biological system are very complex, sometimes other proteases like chymotrypsin, papaine, pepsin are used (Table 1).

Table 1: Protease used for In-gel and In-solution methods (Granvogl et al., 2007)

Protease	Class	Specificity(Cleaves at the)	Optimum pH
Trypsin	Serine protease	C-terminal of **L**&**R**	7.5 – 9.0
Chymotrypsin	Serine protease	C-terminal of **Y**, **W**&**F**	1.5 – 8.5
Proteinase K	Serine protease	C-terminal of hydrophobic aminoacids	7.0
Thrombin	Serine protease	C-terminal of **R**	7.5
Arg C	Cysteine protease	C-terminal of **R**	7.5 – 8.5
Papaine	Cysteine protease	C-terminal of **L**, **R**, **G**, **H**&**Y**	7.0 – 9.0
Pepsin	Aspartate protease	C-terminal of **W**, **L**, **M**&**F**	2.0 – 4.0

3.Methodology for in-gel digestion

The protein spots should be excised from the 1D (SDS-PAGE) or 2DE (two dimensional gel electrophoresis) gels by using a clean surgical blade or from a robotic spot picker. Transfer the protein spots into fresh eppendorf tubes. Prepare all the reagents in the ultrapure water. A protocol for the in-gel digestion is described here but it can be modified depending upon experimental requirements.

3.1. Rehydration and dehydration:

- Theprotein spots (gel pieces) should berehydrated with 100µl of 25mM ammonium bicarbonate (ABC) and vortexed for 5 minutes and solution is discarded.Acetonitrile is an organic solvent; hence it removes the Coomassie brilliant blue stain from the gel pieces.
- 100µl of sol. A (1:2 ratio of 25mM ABC: Acetonitrile)is added for dehydration and vortexed for 5 minutes. After 5 minutes the solution should be discarded.
- Repeat the above steps twice and perform afinal wash with the sol. A.

3.2. Reduction and alkylation (optional):

This step is essential for those proteins, which are having disulphide bonds.

- To the gel pieces add 10mMDithiothreitol (DTT) in 100mM ammonium bicarbonate solution and incubate at 56^0C for 1 hour.
- After 1 hour remove the solution and add 100µl25mM ABC and vortex for 5 min. and then discard the solution.

- Add 100µl of 50mMIodoacetamide (IAA) in 100mM ABC solution. Incubate at room temperature for 30 min.
- Discard the solution and add 100µl of 25mM ABC and vortex for 10 min, then discard the solution. Add 100µl of sol. A to the gel pieces and vortex for 10 min. and then discard the solution.
- Repeat the above step one more time.

In this step addition of DTTresults in cleavage of the disulphide bonds and the addition of IAA results in the alkylation of the reduced disulphide bonds, which prevents the disulphide bond formation. The protein spots obtained from the 2D-gels have undergone reduction (with DTT) and alkylation (with IAA) in the process of 2-DE. So this step is optional for those protein spots obtained from 2-DE method.

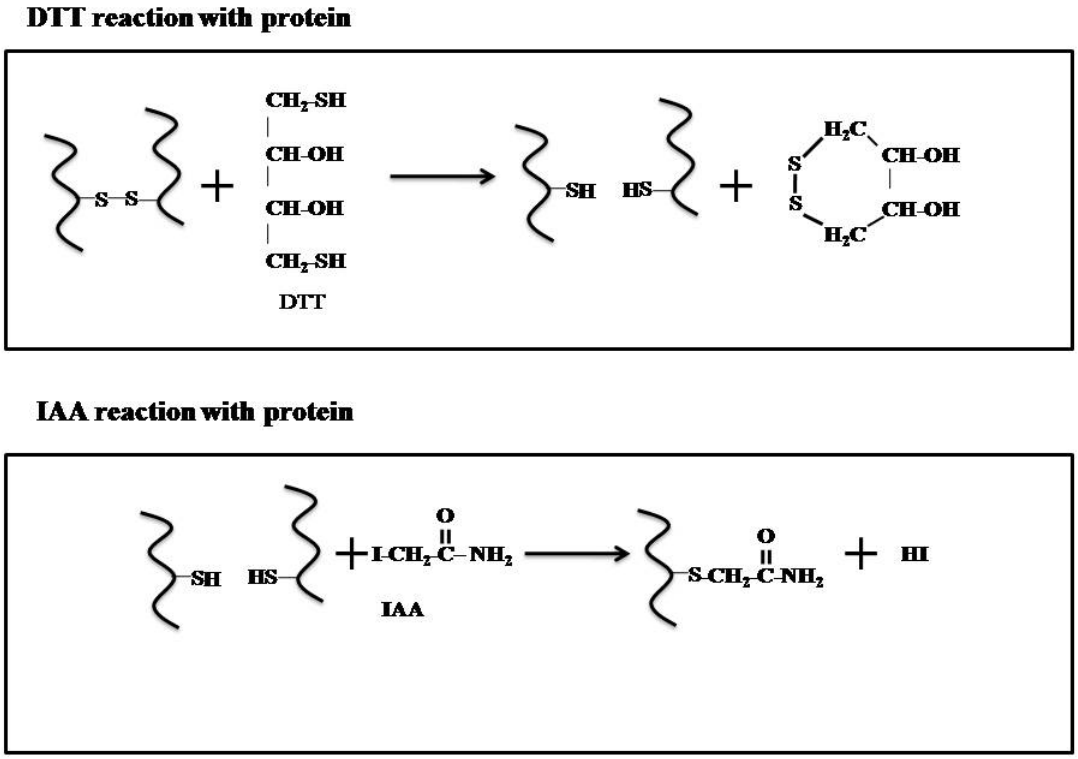

Fig.2. Reduction of disulphide bonds by Dithiothreitol and alkylation of thiol groups by the iodoacetamide.

3.3. Tryptic digestion:

- To the gel pieces add 400ng of trypsin in 50mM ABC.
- Incubate on ice for 30 minutes so that the trypsin is absorbed by the gel pieces.
- Add 50µl of 50mM ABC to the gel pieces and incubate at 37^0C for 16 hours.

3.4. Peptide extraction:

- After overnight incubation with trypsin the proteins in the gel pieces are cleaved into peptides. Collect the solution from the overnight incubated gel pieces into a fresh eppendorf tube.
- Prepare 50%, 60% and 80% acetonitrile (ACN) solution in ultrapure water containing 0.1% TFA (Trifluoroacetic acid).
- Add 100µl of 50% ACN solution to the gel pieces and vortex for 10 min. Then collect the peptides containing solution.
- Add 100µl of 50% ACN solution to the gel pieces and sonicate for 1 min (2 sec. pulse, 1 sec. gap and 25% amplitude). Vortex the sonicated samples (gel pieces with 60% ACN solution) for 10 min and collect the peptide solution.
- Add 100µl of 80% ACN solution to the gel pieces and vortex for 10 min. and collect the peptide extract (Modified from the reference Shevchenko et al., 2007).

3.5. Concentrating the peptide sample:

The extracted peptide sample is subjected to speed vac. for concentrating the peptides. After speed vac. the peptide solution volume should be around 10-20µl. If the peptide sample is completely dried then add 15µl of 50% ACN solution in ultrapure water having 0.1% TFA and vortex for 10 min.

3.6. Zip tipping:

- Zip tipping is done to remove the excess salts from the peptide extracts. Zip tips contain C18reverse phase resins. These resins bind to the peptides, when the

peptide sample is allowed to pass through it. And the peptides are eluted out when elution solution is added to the column.

- The zip-tips are fitted to the 20µl fine micropipette. The C18 resin is activated by aspirating 10µl of wetting solution (100% ACN) and discarding it. This process is repeated for two more times.
- The C18 resin is washed with the washing solution (0.1% TFA in ultra pure water) thrice by aspirating 10µl of washing solution and then discarding it.
- Aspirate the peptide solution and expel it back into the tube. Repeat this process 10-15 times. In this process the peptides bind to the resin.
- Wash the resin by aspirating washing solution (0.1% TFA) and expelling it. Repeat it thrice. In this process salts are removed from the resin.
- Aspirate 10µl of elution solution (50% ACN in ultra pure water containing 0.1% TFA) and expel it into a new tube. Aspirate the same elution solution which was expelled in the earlier step and expel it again into the same tube. Repeat this process 10-15 times. In this process the protein is eluted out of the zip tip.
- Now peptide samples are ready for analysis using mass spectrometry.

3.7. Schematic for in-gel digestion method

Cut the protein spot/ band from the gel

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Add 100 µl of 25 mM ABC and vortex for 5 min. Discard the solution

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Add 100 µl of "Sol. A" and vortex for 5 min. Discard the sol

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Repeat the above two steps twice

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Add 100 µl of 10 mM DTT in 100 mM ABC

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Incubate at 56^0C for 1 hr. on dry bath and then discard the sol

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Add 100 µl of 25 mM ABC, vortex for 5 min. and discard the sol

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Add 100 µl of 50 mM IAA to the gel pieces and incubate for 30 min. in dark condition

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Remove the IAA solution from the gel pieces

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Add 100 µl of 25 mM ABC and vortex for 5 min. then discard the sol

↓

Add 100 µl of "Sol. A" and vortex for 5 min. then discard the sol

↓

Add 100 µl of 25mM ABC and vortex for 5 min. then discard the sol

↓

Add 100 µl of "Sol. A" and vortex for 5 min. then discard the sol

↓

Add 400 ng of Trypsin in 50 mM ABC to the gel pieces

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Incubate on ice for 30 min

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Add 50 µl of 50 mM ABC and incubate at 37^0C for 16 hour

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Collect the peptide sol. from 16hr. incubated gel pieces

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Add 100 µl of 50% ACN in double distilled water having 0.1% TFA

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Vortex for 10 min. and collect the peptide sol.

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Add 100 µl of 60% ACN in double distil water having 0.1% TFA

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Sonicate the gel pieces using 25% amplitude, 2 sec. pulse & 1 sec. gap for 1 min.

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Vortex for 10 min. and collect the peptide sol.

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Add 100 µl of 80% ACN in double distilled water having 0.1% TFA

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Vortex for 10 min. and collect the peptide sol.

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Speed vac. the peptide sol. until the volume is reduced to around 10-20 µl

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Zip-tip the peptide samples

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Spot on MALDI plate and perform MS

Illustration: In-gel digestion

The proteins separated by 2D are analyzed, and spots, which exhibitstatistically significant differences, are excised, processed and taken for mass spectrometric analysis. Prior to the Mass spectrometry analysis it is important to cleave the protein in the gel by trypsin to make them smaller peptides for easy analysis by Mass spectrometry. A working protocol is demonstrated in animation.

4.1. Methodology for In-solution digestion

This procedure is generally used for the LC-MS/MS based experiments. In this method extracted protein isdirectly trypsinized in solution, so it requires less amount of protein sample and easy to alter the experimental conditions.

- To the protein sample add 15µl of 166 mM ABC and 25µlTrifluoroethanol (TFE) vortex and then incubate at 90^0C for 1 hr. and then cool the sample to room temperature.
    - NOTE:Low sample volumes and high concentrations are preferred.
- Add 2.5µlof 200mM DTT solution and incubate at room temperature for 1 hr. The disulphide bonds of the proteins are broken in this step.
- To the above mixture add 12.5 µl of 200 mM IAA and incubate for 1 hr. in dark at room temperature. The free thiol groups on the proteins are alkylated by the IAA.
- Again add 2.5 µl of 200mM DTT solution and incubate at room temperature for 1 hr. In this step excess free IAA will be removed by the DTT.
- Then add 300 µl of double distilled water and 100 µl of 100mM ammonium bicarbonate (ABC). The resulting solution pH should be maintained in between 7.5 - 8.0. Double distilled water dilutes the TFE (denaturant) and the 100mM ABC increases the pH of the protein sample.
- To the protein solution 10 µg of Trypsin should be added, vortexed and incubated at 58^0C for 1 hr. followed by overnight incubation at 37^0C.
- After overnight incubation the trypsin activity is stopped by the addition of 2 µl of formic acid (Ru et al., 2006).

4.2. Schematic for the in-solution digestion method

To the protein solution add 15µl of 166 mM ABC and 25µl TFE

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Vortex and incubate at 90^0C for 1 hr. and cool the sample to RT

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Add 12.5 µl of 200 mM IAA and incubate for 1 hr. in dark at room temperature

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Add 2.5 µl of 200 mM DTT solution and incubate at room temp. for 1 hr

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Add 300µl of double distill water and 100 µl of 100mM ABC

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Adjust the pH of the resulting solution to around 7.5 - 8.0

Add 10 µg of Trypsin, vortex and incubate at 58^0C for 1 hr

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Incubate at 37^0C overnight for protein digestion

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Add 2.0 µl of formic acid to stop the trypsin activity

Illustration: In-solution digestion

In solution digestion is performed in order to analyze all the proteins that are present in the sample. The method usually doesn't require any protein separation. This experiment should be performed on whole proteome, which is to be analyzed. A working protocol is demonstrated in animation.

5. Sample preparation for mass spectrometry

The in-gel digested and concentrated protein samples should be further mixed with proper matrix in order to spot on the MALDI plate to perform MS. These matrices are aromatic compounds, when the laser strikes the matrix-peptide mixture; the matrix molecules absorb the energy and transfer it to the peptides. This results in generation of peptide ions, which travel through the TOF tube, and gets detected by the detector.

In case of LC-MS/MS based methods the protein/peptide samples are allowed to pre-fractionate by using various liquid chromatography methods. The proteins/peptides are enriched in the chromatography columns/chips and are further subjected to MSor MS/MS analysis.

6. References

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