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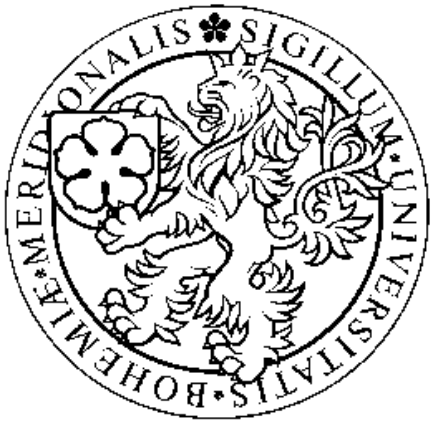

Mass Spectrometry of Proteins

Biological Chemistry

Bachelor Thesis

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Annotation

The topic of this Bachelor thesis is the mass spectrometry of peptides and proteins. First there is a short introduction and an overview about the historical development of mass spectrometry of proteins. Then the devices that are commonly used for analysis are discussed. The last part of the thesis deals with the protein fragmentation and the interpretation of the obtained mass spectra.

Affirmation

I hereby declare that, in accordance with Article 47b of Act No. 111/1998 in the valid wording, I agree with the publication of my bachelor thesis, in full to be kept in the Faculty of Science archive, in electronic form in publicly accessible part of the STAG database operated by the University of South Bohemia in České Budějovice accessible through its web pages.
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May 9th, 2011

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Tamara Buchberger

Acknowledgement

I am grateful to Prof. RNDr. Libor Grubhoffer, CSc. for supervising my Bachelor thesis.
Further I want to thank my family, friends and colleagues for supporting me.

List of Abbreviations

AQUA	Absolute quantification of proteins
BIRD	Blackbody infrared dissociation
CAD	Collision activated dissociation
CID	Collision induced dissociation
Da	Dalton
dc	Direct current
ECD	Electron capture dissociation
ESI	Electrospray ionisation
ETD	Electron transfer dissociation
ExD	Abbreviation for ECD/ETD
FTICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
HILIC	Hydrophobic interaction liquid chromatography
HPLC	High pressure liquid chromatography
IRMPD	Infrared multiphoton dissociation
LC	Liquid chromatography
MALDI	Matrix assisted laser desorption/ ionisation
MS	Mass spectrometry
MS^n	Tandem mass spectrometry
PTM	Posttranslational modification
rf	Radio frequency
SAM	Self-assembled monolayer
TOF	Time-of-flight

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1 Introduction

Mass spectrometry (MS) and tandem mass spectrometry (MS/MS) turned out to be very powerful tools for the identification of the primary structure of peptides and proteins up to research of whole proteomes. Mass spectrometers measure the mass/charge (*m/z*) ratio of either whole proteins or protein complexes or fragment ions. These fragment ions can be derived from enzymatic or chemical digests, from gas-phase activation of protein ions (top-down sequencing) or from gas-phase activation of mass-selected peptides (bottom up sequencing). The gained information is then compared to the results of "theoretical" digests or "theoretical" fragmentation. This comparison for the identification requires a substantiated knowledge of the interplay between the mass spectrometry instrument (ionisation, activation and detection of ions) and the chemistry of gas-phase peptides (which bond breaks, with which rate and under which conditions, as charge state, peptide size and sequence). The great success of protein sequencing was possible with the introduction of "soft" ionisation techniques, namely electrospray ionisation (ESI) and matrix assisted laser desorption/ionisation (MALDI) [1]-[3].

The four great advantages of mass spectrometric sequencing include the high sensitivity, the rapid speed of the analysis, the large amount of information that is generated and the ability to detect post-translational modifications [1]:

- *Sensitivity:* The detection of routine analyses is in the range of femtomole quantities of a peptide and with careful application even attomole levels can be achieved [4], [5]. Therefore accumulation of material that can be sequenced is no longer necessary.

- *Speed:* Mass spectrometric experiments can be carried out up to a several-proteins-per-hour level and can be carried out routinely. This is especially possible due to the introduction of liquid chromatography (LC) coupled to MS. Quite as fast as LC/MS is MALDI-TOF with the advantage of less complex data [6].

- *Amount of information:* The amount of data that is produced enhances the confidence of the identification and it allows the identification of complex protein mixtures.

- *Post-translational modifications:* Modifications can be easily identified from the obtained *m/z* ratio by tandem mass spectrometry. The ionisation is either done by ESI or MALDI.

1.1 Amino Acids, Peptides, Proteins

All amino acids consist of a central carbon atom with an attached hydrogen atom, a carboxylic acid group and a primary amine group. The fourth substituent is generally described as R. In Figure 1 the different types of R for the 20 amino acids are shown. Investigation showed that there exists another amino acid called selenocysteine but due to its rare appearance it is not of further interest. A fundamental property of all proteins is that all proteins consist of only those 20 amino acids, varying in the sequence and the number of amino acids. Proteins are formed by a condensation reaction between the primary amine group of one amino acid and the carboxylic acid group of another amino acid. The result is an amide bond between the two amino acids. The first amino acid of a sequence has a free primary amine left; therefore it is called the N-terminus of the protein. The last amino acid has a free carboxylic acid group and is called C-terminus of the protein. Peptides can be seen as small proteins. There are no specific limits but peptides might have up to 20 amino acids and proteins have more than 20 amino acids. The identification of the sequence is not only important for the identification of a protein but might also be used for correlation with the corresponding DNA. This is of great importance for the comparison of genomic and proteomic researches. Whereas the genome defines all proteins that could be expressed, the proteome represents all the proteins that are actually expressed [7]

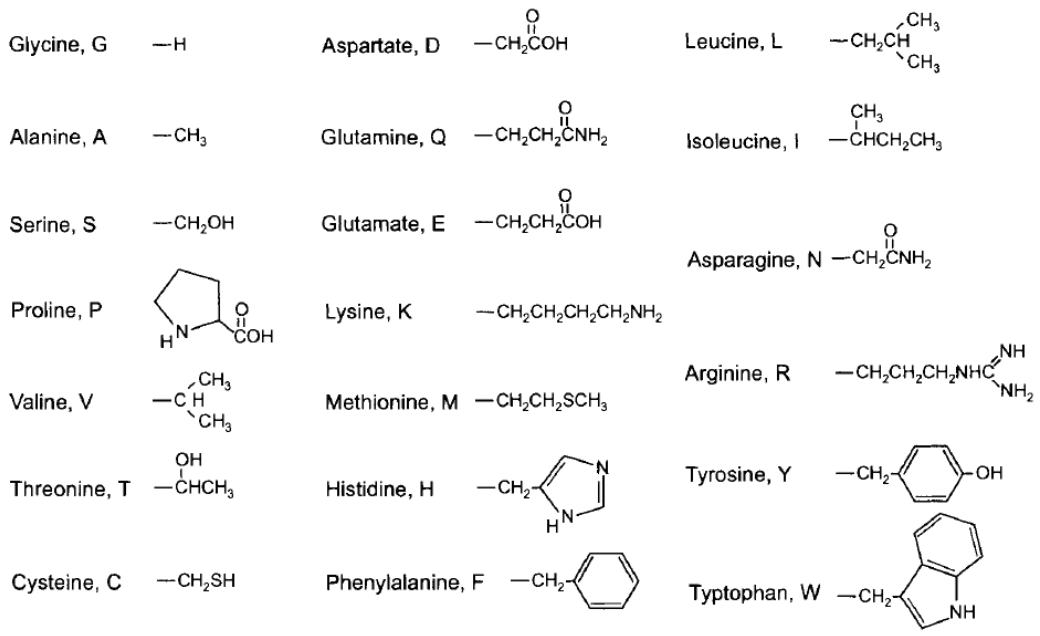

Figure 1: Structure of the side-chain of the 20 genetically encoded amino acids and their one-letter code (proline is drawn fully)

2 Historical Overview of the Development of Mass Spectrometry

First attempts for the determination of the amino acid sequence in peptides were carried out by Edman using the so-called Edman degradation in the early 1950s [8]. From the early 1960s on first mass spectrometric experiments for peptide sequencing have been carried out. The greatest problem was the vaporisation and the ionisation of the big proteins. In the beginning electron ionisation has been the method of use. Later fast atom bombardment (FAB) has been utilised. The great breakthrough in mass spectrometry goes along with the commercialisation of ESI and MALDI as soft ionisation techniques in the 1990s.

2.1 Edman Degradation

The Edman degradation consists of several circles shown in Figure 2. In each circle the N-terminal amino acid of the protein is cut off. The cleaved amino acid can now be identified. Originally Edman used a thin layer chromatography with UV-detection [9], whereas others used gaschromatography with a flame-ionisation-detector. The biggest advance in this field was the introduction of the high-pressure-liquid-chromatography (HPLC) for the analysis of the removed amino acid [1], [2]. The detection can also be done by a mass spectrometric analysis. The critical parts of the Edman degradation are the yields of degradation steps, the sensitivity, which means how much protein is necessary, and the time that is needed for repeating the cycles. Over the years lots of improvements have been made that increased the utility and the sensitivity of the Edman degradation. The first step was the automation of all reaction steps in 1967 by Edman [9]. To overcome the problem of losing analyte the next step was the immobilisation of the protein on a solid support.

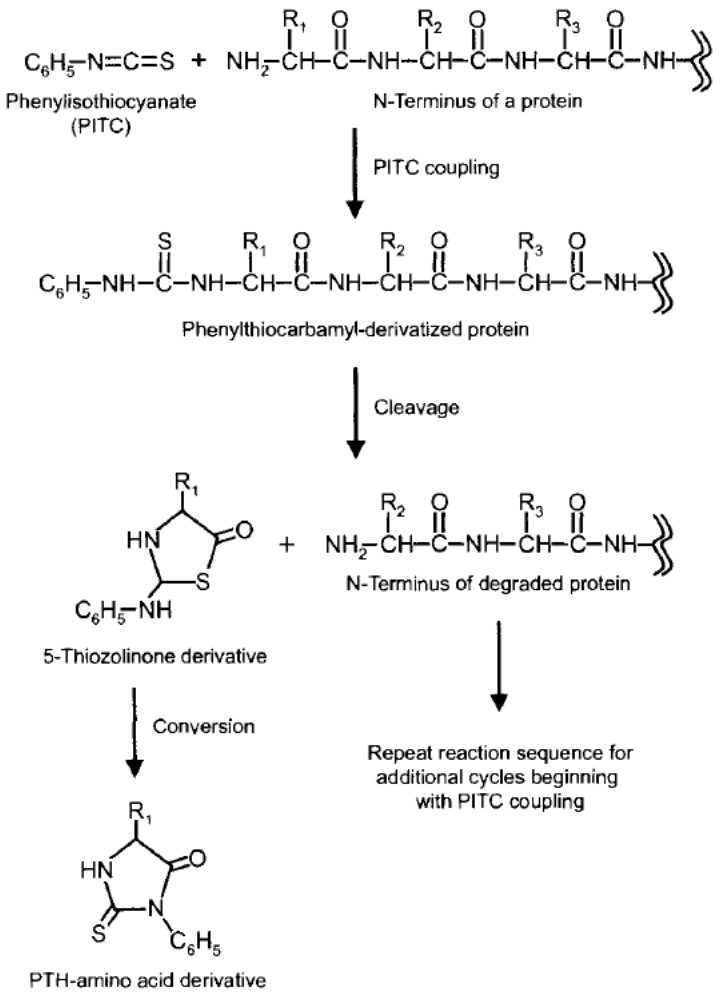

Figure 2: Scheme of the Edman reaction

2.2 Mass Spectrometry

As already mentioned earlier the problem at the beginning of using mass spectrometry for protein sequencing was the generation of gas-phase ions of peptides or proteins. In general the development of MS for the sequencing can be divided into three periods. The first period extends from the early 1960s when the first MS experiments for sequencing took place up to the introduction of fast atom bombardment in 1981. During this first period electron ionisation was the method of choice for the ionisation of small peptides. Usually it accompanied the Edman degradation. The second period started with the employment of FAB and extends up to the 1990s. This method has already many advantages of the modern MS techniques but the sensitivity is hardly better than of the Edman degradation. With the

introduction of ESI and MALDI as soft ionisation techniques for tandem MS experiments in the 1990s the third period started [1].

During the first period usually derivatives of amino acids have been analysed. This was necessary to enhance the volatility of the peptides and furthermore made the fragmentation pattern more informative. The first experiments were the analysis of the silk protein and actin. Before the MS experiments were carried out the proteins were hydrolysed with acid and then purified by gaschromatography (GC) [1], [2].

Despite the first successes during the first period it was still a problem to get gas-phase ions of greater peptides. In 1981 FAB was introduced for the generation of gas-phase ions of polar analytes. For FAB experiments a sample is dissolved in a viscous, non-volatile matrix and then bombarded with atoms that have several thousand electronvolts of kinetic energy. As FAB also belongs to the so-called “soft” ionisation techniques only little fragmentation is observed. This lack of fragmentation combined with the high background signal of the matrix limits the method to the determination of molecular weights of peptides without sequence information.

A great step forward to modern MS analysis of proteins during the second period was the introduction of the first tandem MS experiments with collisionally induced dissociation (CID). Devices are a magnetic sector (B) – electric sector (E) geometry or a triple quadrupole. The combination of FAB and tandem mass spectrometry allowed the sequencing of many proteins during the 1980s. Furthermore it was possible to detect post-translational modifications.

As already mentioned the most recent period in the development of mass spectrometry began with the commercialisation of ESI and MALDI in the 1990s. Like fast atom bombardment ESI and MALDI are also “soft” ionisation techniques so that only little fragmentation occurs. Therefore tandem mass spectrometry is necessary to gain peptide sequence information. The advantages of ESI are that it can be easily adapted to use it with in-line liquid chromatography and its sensitivity is several orders-of-magnitude better than FAB. Although MALDI cannot be used with in-line liquid chromatography it has exceptional sensitivity.

The fragmentation with interpretable ion spectra could be obtained from the collisionally induced dissociation of the multiple charged peptide ions. Also tryptic digests played an important role in the production of peptide fragments. Another important part of the development of protein sequencing was the increasing amount of known DNA sequences that could be correlated with the amino acid sequence of the proteins, e. g. from the Human Genome Project. The progress of relevant software such as databases and the software for matching sequences (such as the FASTA programs) goes along with the knowledge of DNA sequences. These developments altogether made it possible that the sequencing of a protein turned from manual chemical technique into a highly automated instrumental technique [1], [3].

3 Mass Spectrometers

In Figure 3 the basic components of a mass analyser are shown. It consists of an inlet, an ion source, a mass analyser, a detector, a vacuum system, an instrument control system and a data system. The most important parts are the inlet, the ion source and the mass analyser as they define the type of instrument. In the following sections selected parts of a mass analyser will be discussed [1].

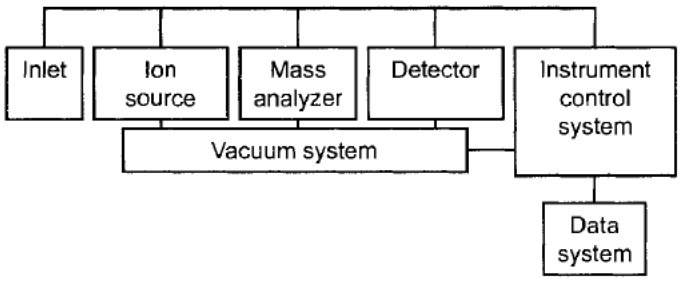

Figure 3: Basic components of a mass analyser

3.1 Ionisation

As already mentioned before the most challenging step in mass spectrometry of proteins is the formation of gas-phase ions. Nowadays this problem has been solved using electrospray ionisation (ESI) especially in combination with chromatography or matrix assisted laser desorption/ ionisation (MALDI) [1].

3.1.1 Electrospray Ionisation

Figure 4 shows a model of the basic process that is going on in the ion source. The – mostly acidic – solution with the peptides is sprayed through a small needle to which high voltage is applied. The acid in the solution assures the positive charge of the peptides. Due to the voltage a Taylor cone is formed. It is assumed that from the Taylor cone small droplets of the

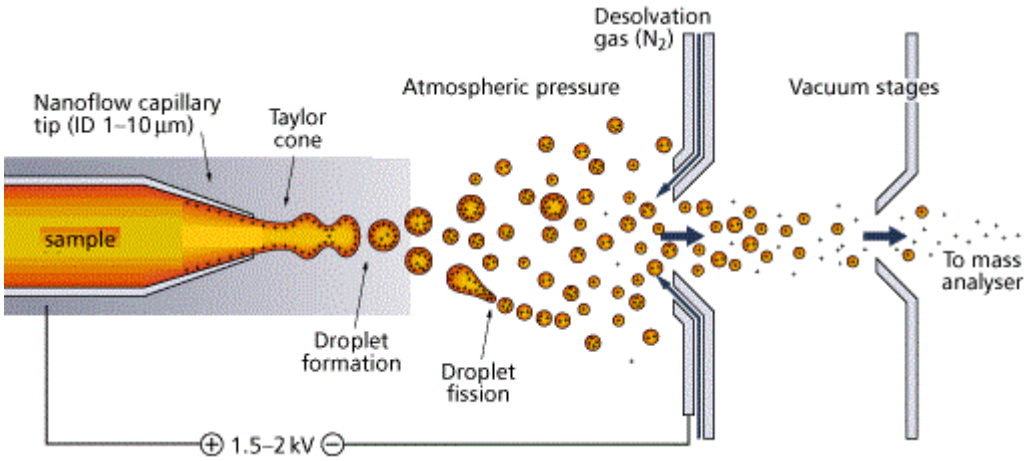

Figure 4: Scheme of an ESI ion source

solution are sputtered. The positive charge causes a movement of the droplets towards the negatively charged part of the instrument. During the movement continuous evaporation causes a shrinking of the droplets. At a certain point it is assumed that the positive charge on the surface of the droplets is too high and causes the explosion of the droplets – so-called Coulomb explosions. The evaporation of the solvent is forced by a gas flow and heat. At a certain point the splitting of the droplets will repeat until bare protonated peptides are released into the gas-phase and move directly into the mass analyser [1].

One important aspect of ESI is that under acidic conditions all basic sites are protonated. Peptides possess a positive charge at their N-terminal end and on the side groups of lysine, arginine, and histidine. The efficiency of protonating all these basic sites is very high and as a result also the sensitivity. The protons on the peptides are important as they drive the fragmentation process and are achieved by the addition of acid to the sample solution. Another great advantage of ESI is its compatibility with HPLC because of the good spray properties of water/solvent mixtures, e. g. methanol or acetonitrile [1].

3.1.2 Matrix Assisted Laser Desorption/ Ionisation

Another ionisation technique that is often used for peptide sequencing is MALDI. A scheme of such an ion source is shown in Figure 5. For this method peptides are dissolved in a solvent that absorbs UV light and then placed on a probe. As the matrix crystallises the peptides are

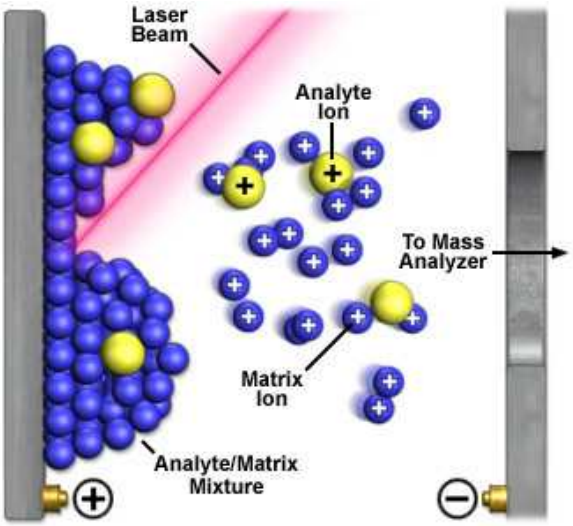

Figure 5: Scheme of a MALDI ion source

included into these crystals. Then a pulsed UV laser vaporises small amounts of the matrix with the included peptides. The protonation of the peptides occurs due to the acidic environment caused by the matrix or some additives. The matrix usually contains aromatic acids or aromatic carbonyl compounds with a high absorption coefficient for the laser and to have the ability to convert the laser energy into heat. Due to the generation of the ions in discrete amounts MALDI is usually used in combination with time-of-flight analysis. An advantage of MALDI compared to ESI is that it is more insensitive against pollutants, especially urea, sodium dodecyl sulfate or other detergents and glycerol. Further advantages are the capability of transferring very large proteins into the gas phase and the ability to predominantly produce singly charged ions. Because of its non-continuous working MALDI is typically not used in combination with HPLC. In common MALDI experiments concerning proteome research are applied directly to the output of tryptic digests [1].

3.2 Mass Analyser

One characteristic that all mass analyser have in common is that they measure *m/z* ratios. Nowadays a great variety of mass analysers is available, including quadrupole mass filter, ion trap, time-of-flight, magnetic sector, and ion cyclotron resonance. Two important factors for their characterisation are obvious: the *m/z* resolution and the *m/z* range of ions that can be

measured. The *m/z* resolution is defined as $m/z/\Delta m/z$, where $\Delta m/z$ can be defined for example as peak-width at half-height. The higher the resolution the more narrow are the peaks and further peaks with similar *m/z* can be resolved. Also the precision is better as the centre of a *m/z* ratio is better defined. This is important for the determination of the charge state of the ion. The *m/z* range determines the range of ions that can be analysed. Mass analysers with a range up to 2 000 Da are sufficient for peptide sequencing with typically double or triple charged ions. In tandem MS the mass ranges usually from about 50 Da to the molecular weight of the peptide. For peptides with greater *m/z* than 2 000 Da only double or triple charged ions can be observed. In the following section the focus will be on quadrupole mass filters, ion trap, and time of flight, as they are commonly used in peptide sequencing [1].

3.2.1 Quadrupole Mass Filters

A scheme of a quadrupole mass filter can be seen in
Figure 6. It is composed of two pairs of electrically connected rods. A combination of radio frequency (rf) and direct current (dc) voltages is applied to each pair of rods which causes a complex, oscillating movement of the ions while travelling trough the mass filter. As a result selected *m/z* can pass the filter on a stable trajectory while others cannot. A mass spectrum can now be acquired by ramping of the applied fields so that increasing *m/z* can pass the filter and reach the detector. Typical rates for such measurements are 2 000 *m/z* per second which is suitable in combination with liquid chromatography. Another possibility to operate a quadrupole mass filter is to transmit ions of all *m/z* by applying only an rf field. This is of interest where transmission without filtering is needed, for example in collision cells of tandem mass spectrometers [1].

A great limitation of quadrupole mass filters is the amount of ions that is transmitted to the detector when it is scanned from low *m/z* to high *m/z*. For continuous ion sources like ESI, the majority of the produced ions are directed out of the instrument. This limits the sensitivity significantly compared to ion traps or time-of-flight mass analysers [1].

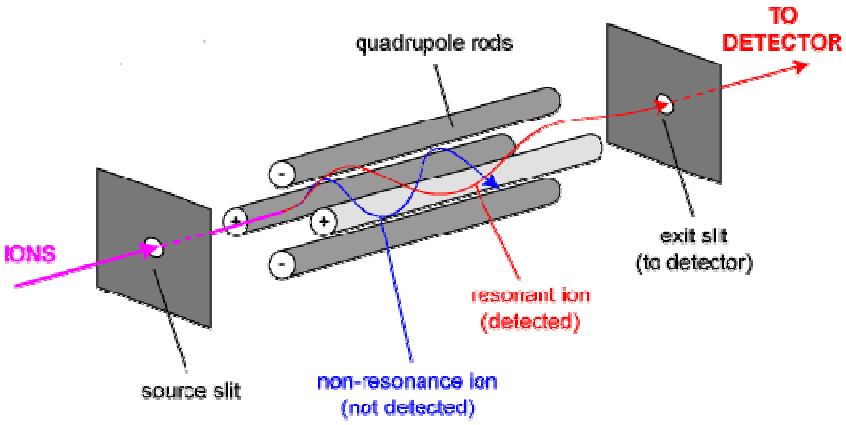

Figure 6: Scheme of a quadrupole mass filter

3.2.2 Ion Trap Mass Analysers

An ion trap is similar to a quadrupole mass analyser in the rf-mode (Figure 7). The field that is applied to the ions catch all *m/z* causing them to oscillate in the mass analyser. Then the rf-voltage is subsequently increased so that increasing *m/z* are ejected out of the trap. This provides the basis for the extremely good sensitivity of an ion trap. Beside the small size of this type of mass analyser, which allows a small distance from the ion source to the analyser, the trapping makes an efficient use of the ions possible. Mass resolution and *m/z* rage of ion trap are similar to those of quadrupole mass analysers. A unique characteristic of ion traps, however, is that they have low *m/z* limitations, which is especially interesting in tandem MS experiments. In these cases low *m/z* are produced which other mass analysers cannot record [1].

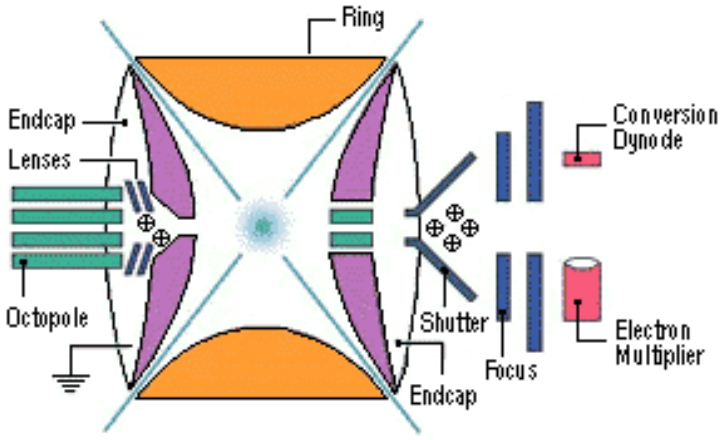

Figure 7: Scheme of an ion trap mass analyser

3.2.3 Time-Of-Flight

For the *m/z* analysis in a time-of-flight (TOF) mass analyser the ions are given a fixed amount of kinetic energy by acceleration in an electric field. The applied voltage is about +20 to +30 kV for positive ions such as peptides. After acceleration the ions enter a field-free region where they travel with a velocity inversely according to their *m/z*. That means that ions with low *m/z* travel faster than ions with high *m/z*. The time they need to travel through the field-free region is then correlated with the *m/z* of the ion (Figure 8). The resolution depends on several factors, such as the time span of the ionisation event, the energetics of the produced ions, the length of the flight tube, and the accelerating voltage. The ions that are produced have to enter the TOF mass analyser in a pulse. The length of this pulse can produce a variance in the flight time of the ions and further cause a lowered *m/z* resolution. Therefore a TOF mass analyser is ideally combined with a matrix assisted laser desorption/ ionisation, that produces ions in a short pulse, or with an electrospray ionisation with electrostatic gates. The variation in the initial velocities of the ions restricts the resolution most. This is a result of the vaporisation and ionisation process. The velocities spread and as a consequence – after acceleration – the flight time differs. This effect can be minimized by using longer flight tubes and higher acceleration voltages. Longer flight tubes mean longer flight time and the relative contribution of Δt produced by the kinetic energy spread to the overall flight time is decreased proportionally. A higher acceleration voltage gives ions a greater amount of kinetic energy so that the relative contribution of the kinetic energy derived from the ionisation process is reduced. Further a delayed extraction of the ions into the flight tube or the use of a reflectron (Figure 9) help to overcome the problem of the initial velocity. The reflectron is an electric field that reverses the flight path of the ions and focuses ions with the same *m/z*. Ions with the same *m/z* penetrate the reflectron with a different degree. Ions with higher velocity penetrate farther and, therefore, spend a longer time in flight tube than ions with lower velocity that penetrate the reflectron to a lower extent. As a consequence velocity differences are compensated. Another effect is that a reflectron extends the flight path. The mass range of a TOF mass analyser is essentially not limited and their sensitivity is extremely high [1].

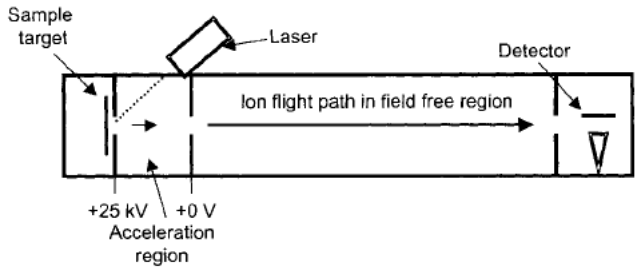

Figure 8: Scheme of a linear time-of-flight mass analyser

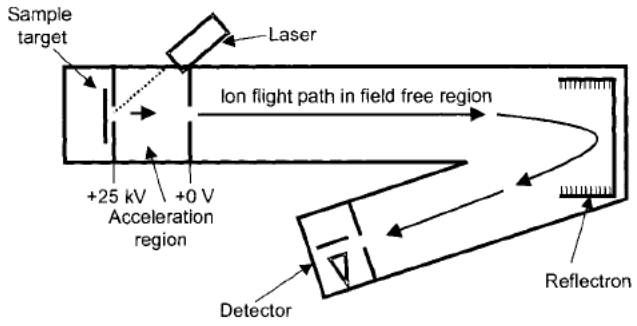

Figure 9: Scheme of a reflectron time-of-flight mass analyser

3.3 Tandem Mass Spectrometry

The special feature of tandem mass spectrometry is that a specified *m/z* can be isolated for further analysis. This analysis includes a fragmentation and is followed by a determination of the fragment *m/z*. Tandem mass spectrometer systems can be divided into two classes: tandem-in-space and tandem-in time [1].

3.3.1 Tandem-In-Space

The mean characteristic of tandem-in-space instruments is that they have more than one mass analyser, and each of them performs on a different stage of the experiment. A typical example is the so-called “triple” quadrupole, which consist of three single quadrupoles one after another. The quadrupole in the middle acts as collision cell, where the selected *m/z* are

fragmented. Modern instruments use an octapole lens system instead of the quadrupole as a collision cell. The fragments are then transmitted to the third quadrupole for analysing [1].

Another system uses a quadrupole as first mass analyser and a time-of-flight as second mass analyser. An octapole lens system is again used as collision cell. The analysis benefits from all advantages from the Q-TOF that have already been mentioned before [1].

3.3.2 Tandem-In-Time

Compared to tandem-in-space instrument systems tandem-in-time instruments use only one mass analyser, which makes it necessary that the system can trap ions. An ion trap or ion cyclotrone resonance instruments would be typical examples for tandem-in-time instruments. In an ion trap first all *m/z* are collected. Then a specified *m/z* is selected by modifying the rf voltage and all other *m/z* are removed from the trap. Afterwards an rf pulse is applied to the selected *m/z* so that collisions between the mass-selected ions and the gas molecules occur. Finally all *m/z* of the produced fragment ions are analysed. These experiments are called "MS^n" experiments where n denotes the number of mass-analysis stages [1].

4 Fragmentation and Interpretation

In this chapter the fragmentation and the interpretation of product ion spectra will be discussed. The condition for a successful protein fragmentation is an appropriate activation of the gas-phase ions, where enough energy is transferred to the ions so that bond cleavage occurs. For the correct interpretation of the resulting product ion spectra fundamental knowledge about the structure of the protonated peptide, the reaction mechanism of the fragmentation and resulting structure of the fragments is necessary. Interpretation of routine analysis of known proteins is nowadays supported by database research programs. These programs utilise unprocessed or only little processed product ion spectra to compare them with large databases containing theoretical spectra of proteins or translated gene sequences. No database search program can, however, replace the understanding for the produced result. The programs just give scores to rank the possible matches. Furthermore the databases are not complete and the search might fail for several reasons such as posttranslational modifications (PTMs) [1].

4.1 Ion Activation

The most widely used ion activation method is the collision induced dissociation (CID) or also called collision activated dissociation (CAD). Besides this method lots of efforts are made in the development of other ion activation methods such as surface induced dissociation (SID), electron capture dissociation (ECD), electron transfer dissection (ETD), infrared multiphoton dissociation (IRMPD), or blackbody infrared dissociation (BIRD). Motivations for development of new techniques are their orthogonality to CID, the possibility of understanding the energetics and mechanism of peptide dissociation and the enhanced probability of more structural information by the combination of two techniques [11].

The principle of CID is the collision between the precursor ion and a neutral target gas. These collisions are accompanied by an increase of the internal energy and after redistribution of the energy it leads to bond dissociation. The mechanism behind is assumed to be a two-step process, where the excitation and the decomposition are separated in time. The activation is generally assumed to be orders of magnitude faster than the dissociation. The second part –

the unimolecular dissociation of an excited ion – can only occur if the collision energy is high enough to overcome bond energy. Generally the method can be divided into two categories. Low-energy collisions, that are common in quadrupoles and ion trapping instruments, occur in the range of 1 – 100 eV. N_2 or Ar is used as target gas, which is usually heavier than the target gas in high-energy collision. This provides an adequate transfer of energy even in low-energy collision. However, the collision energy in high-energy collisions is at kiloelectronvolt range and occurs in sector and TOF/TOF systems. Only in these instruments a precursor ion with a high translational energy can be provided. In common the target gas is helium, but collision yield might be increased by using Ar or Xe. The resulting spectra of both methods differ greatly. Generally it can be assumed that the lower the energy is the more rearrangement reactions compared to direct bond cleavages occur. The threshold energy for several reactions is not reached in low-energy collision resulting in less fragmentation. High-energy collision leads to the formation of *v* and *w* ions, whereas low-energy collisions produce *b* and *y* ion. The nomenclature of the product ions will be described in the following sections [10], [11].

The working principle of SID is similar to CID. The main difference between the methods is that instead of the gas in CID a solid surface is used as collision target. Through the collision with the surface the kinetic energy of the ion is transferred into potential energy. As a result it is activated, which is followed by the dissociation. The idea behind is that as the target is “infinite” the conversion of energy should be much more efficient in SID compared to CID. According to the classification of collision events SID reactions fall in the “hyperthermal” regime. This means energy between 1 and 100 eV. In this regime collision energies are in the order of or greater than chemical bond energies and therefore allow bond dissociation and rearrangement reactions. If an ion hits the wall it can scatter in an elastic, and inelastic or chemical reactive fashion. Elastic scattering leads to a reduction of translational energy and further to a depositing of the ions on the surface. The most common cases, however, are inelastic scattering and chemical reactions. Chemical reactions are unwanted side reactions which lead to a reduced efficiency and sensitivity. In general it can be said that the activation mechanism of CID and SID are very similar, that means that SID is also a two-step process. The first step is the collision of the ion with the surface, forming an internally excited ion. This undergoes then unimolecular dissociation. One great advantage of SID over CID is the narrow internal energy distribution of the excited ions. The transferred energy varies with the changes in the impact energy. This makes identification of fragmentation pathways easier.

The amount of transferred energy depends on several factors such as surface composition, collision energy, ion structure or incidence angle. The narrow energy distribution might bear potential for identification of isomers. Further advantages of SID are reproducibility with good signal-to noise-ratios. And compared to CID no additional gas pumping station is required since no extra gas is necessary. Still there are several processes – especially in combination with metal surfaces used for collision – competing with the activation of the ions like neutralisation, chemical sputtering from the surface and ion-surface complex formation reaction. Up to 90 % of the precursor ions are lost with the most metal surfaces. Recently surfaces that are modified with a fluorinated self-assembled monolayer (SAM) of thiolate have been developed. They have a high ionisation potential and are widely resistant to surface damages. SID seems to be a promising method for activation of high-mass peptides especially in combination with FTICR-MS as no additional gas for collision is required [11].

Another activation method is ECD which is based on the capture of low-energy electrons by multiple charged ions. This ends in a reduction of charge and subsequent fragmentation. Singly charged ions cannot be fragmented or detected as their only charged is neutralised after capturing an electron. The fragmentation pathways differ greatly compared to CID as cleavages in ECD are governed by radical ion species. Typical capture sites are carbonyl groups or disulfide bonds, which are not necessarily the charge sites. Compared to all other methods the energy is not redistributed over the whole molecule and therefore not the weakest bondst are preferentially broken. As a result strong backbone N-C_α bonds are cleaved forming *c'* and *z*-type ions. Further also disulfide bonds, which are not broken using other activation methods, are preferentially broken. As a consequence ECD offers the great possibility of the identification of posttranslational modifications and even noncovalent interactions are stable und ECD conditions. With the special application of hot-ECD *w*-ions through side-chain cleavage are observed. These fragments are important in the identification of the isomeric amino acids leucine and isoleucine. The main advantage is the ability to fragment very large peptides (about 40 kDa) at many sites. Usually ECD is used in combination with FTICR mass spectrometer [11]-[14].

The working principle of ETD is similar to ECD. ETD fragments peptides by transferring an electron from a radical anion to the protonated peptide. Also the fragmentation pathways are similar to ECD which means that is mostly used for the identification and characterisation of posttranslational modifications, namely phosphorylation, sulfonation, glycosylation,

nitrosylation, disulfide bond, methylations, and acetylations. That means that ETD shows strong backbone fragmentation producing a complete or almost complete series of ions while PTMs are preserved. The main advantage of ETD over ECD is that it can be used in combination with rf quadrupole trapping devices, which are low-cost, low-maintenance, and widely accessible compared to FTICR mass spectrometers which pushed the breakthrough of the ExD technology [14]-[15].

Another possibility of activation is infrared multiphoton dissociation (IRMPD). This technique has been used for a long time for study of small molecules. The increased usuage for the analysis of peptides can be traced back to the rising popularity of trapping instruments. These instruments can trap the peptides for a long time which allows the peptides to catch enough photons to overcome bond energies. The requirement for satisfying fragmentation is that the precursor ion must be able to absorb energy in the form of photons, producing excited states above the threshold for dissociation. The energy that is gained must overcome the energy lost by photon emission as well as deactivation by collision. The advantage of IRMPD over CID is that the trapping conditions do not have to be altered during the whole process. Generally IRMPD is rather nonselective which might be disadvantageous as all trapped ions undergo fragmentation. As a result also product ions obtained from precursor ions undergo additional fragmentation which makes it difficult to identify fragmentation pathways. IRMPD is ideal for the use in combination with FTICR MS as no additional gas is needed. However, the costs of this technique are high and there might be problems with the identification of fragmentation pathways [11].

The last activation method that will be presented is blackbody infrared radiative dissociation (BIRD). The activation of the ions is accomplished by heating in the ICR cell. The high temperature causes the trapped ions to absorb blackbody photons that are emitted from the wall of the cell. There are two basic requirements for the mechanisms to work: the ambient low pressure and the time-scale of observation is long enough. This is the reason why BIRD is the slowest activation method among all the others with heating times up to several minutes. As a logical consequence it is not compatible with HPLC or CE. Yet this technique is the only one with a direct thermal analogy for calculation of kinetic parameters [11]. Further it is useful in the analysis of large protons in combination with FT-ICR [12].

4.2 Fragmentation Chemistry

For the interpretation of product ion spectra it is essential to have knowledge about the structure of the protonated peptides and the fragmentation pathways. In this section the fragmentation of multiply charged peptides from electrospray ionisation activated by CID will be discussed. The basic nomenclature of product ions can be seen in Figure 10. The *a*, *b* and *c*-ions all contain the N-terminus of the peptide, whereas the *x*, *y* and *z*-ions all contain the C-terminus of the peptide. This section will focus on the *b*-ions and the corresponding *y*-ions as they are the most common ions produced by combination of ESI with CID [1]. Figure 11 displays the fragments that might be achieved from a side chain cleavage of the amino acid valine [2]. The side chain cleavage allows the distinction between isoleucine and leucine.

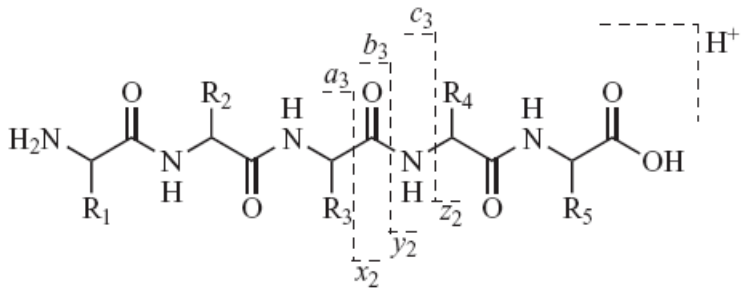

Figure 10: Nomenclature of common product ions

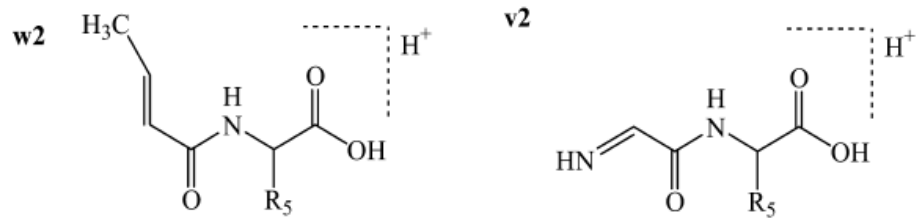

Figure 11: Structures achieved from side chain cleavage of amino acid valine as residue 4

In general it can be said that in a positive operating mode of MS the basic sites of the peptides will be protonated. These sites include the less basic N-terminal amine and the more basic side chains of the amino acids lysine, arginine, and histidine. The proton is attached very strong to the more basic side chains than to the N-terminal amine. Therefore the proton on the

N-terminus will be able to move by internal solvation, which is also called the mobile proton hypothesis. This migration is important because it allows a variety of protonation sites within one peptide which directs the fragmentation. The fragmentation is dramatically dependent on the charge state of the protein and the presence of one or more basic amino acid residues. In a singly charged peptide with a mobile proton the charge can be on each of the oxygens of the amide bonds producing a series of product ions. In a double charged peptide one proton is fixed to the basic residue while one proton is mobile. Without this mobile proton only limited fragmentation is observed as the other proton cannot move. As a result, the most complete fragmentation is seen by fragmenting the highest possible charge state of a peptide. In higher charged peptide the fixed protons gain important relevance as they alter the migration of the mobile proton. The mobile proton would not tend to localise at amide bonds that are nearby the fixed protons. As a consequence only little fragmentations at those positions are observed. Therefore, in higher charged peptides the amount of sequence information depends on the position of the basic residues.

This charge-site-directed fragmentation, as it is called, can occur through a number of pathways but a major pathway that is induced by low-energy collisionally induced dissociation is shown in Figure 12. The singly charged peptide ATSFYL is chosen as an example. The fragmentation proceeds through a cyclic intermediate that fragments by one of the two reactions. Reaction I forms the *b*-ion with the charge remaining at the N-terminal fragment. Figure 12 illustrates the formation of the b_3-ion and analogous reactions would occur for the other amide bonds except for the formation of the b_1-ion because the required cyclic intermediate cannot be formed. Reaction I dominates the product ion spectra compared to reaction II, where the charge remains on the C-terminus forming a *y*-ion, is less favourable. For a doubly charged peptide the situation is similar with one exception: the results are either a singly charged *b* and *y*-fragment or one doubly charged *b* or *y*-ion fragment. These are just the most common fragmentation pathways. There might be also other reactions such as the formation of an *a*-ion by loss of CO. Table 1 lists the residue masses of the 20 amino acids and a selected number of modified amino acids. These residue structures are central to the interpretation of product ion spectra [1].

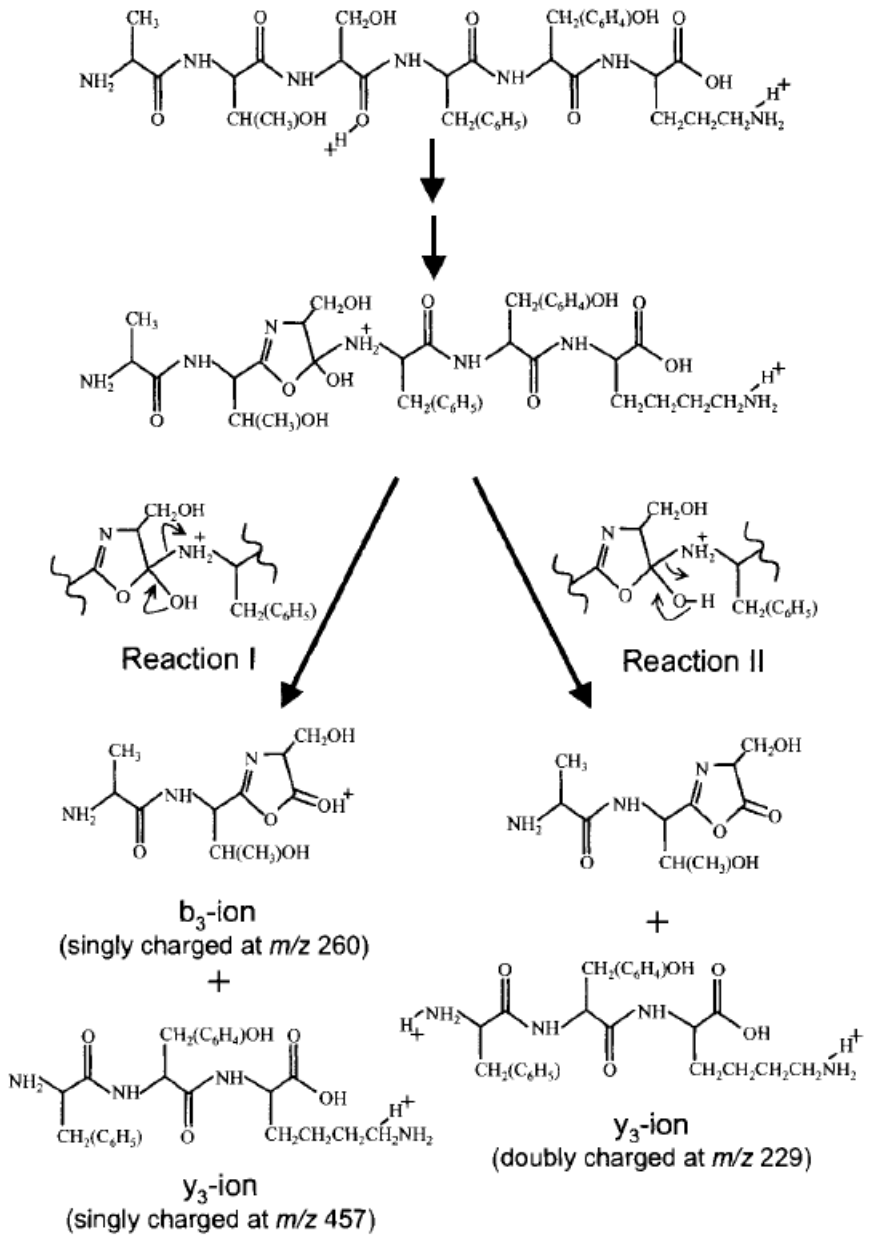

Figure 12: The major fragmentation pathway for the low-energy collisionally induced dissocation (formation of *b* and *y*-fragments)

Table 1: Residue masses of the 20 amino acids and selected modified amino acids

Amino acid	One-letter code	Residue mass (Da)	Immonium ion (m/z)
Glycine	G	57.02	30
Alanine	A	71.04	44
Serine	S	87.03	60
Proline	P	97.05	70
Valine	V	99.07	72
Threonine	T	101.05	74
Cystine	C	103.01	76
Leucine	L	113.08	86
Isoleucine	I	113.08	86
Asparagine	N	114.04	87
Aspartate	D	115.03	88
Glutamine	Q	128.06	101
Lysine	K	128.09	101
Glutamate	E	129.04	102
Methionine	M	131.04	104
Histidine	H	137.06	110
Oxidized Methionine	Mo	147.04	120
Phenylalanine	F	147.07	120
Arginine	R	156.10	129
Carbamidomethylcysteine	C*	160.03	133
Tyrosine	Y	163.06	136
Acrylocysteine	C[a]	174.04	147
Tryptophan	W	186.08	159

4.3 Interpretation of Product Ion Spectra

This section focuses on the interpretation of the product ion spectra especially of tryptic digests. It is based on the recognition of the *b*- and *y*-ion series and the calculation of the residue masses. It has to be kept in mind that this is an iterative process which produces a mathematical solution. It should explain the most common but also the low abundance ions, it should be consistent with the digest and the possible charge state of the observed fragment ions [1].

The residue masses can be found in Table 1. Rounding errors have to be taken into account and someone has to be aware of the fact that these errors sum up and as a consequence the calculated masses might differ greatly to the observed masses in the spectra. This is of special interest when masses of amino acids or fragments overlap, e.g. glutamine and lysine. These amino acids can only be distinguished if high mass resolution is used. The amino acids

leucine and isoleucine are not distinguished as they have identical residue masses and they are treated as one amino acid with the one-letter abbreviation X. In Table 1 additionally the masses of the appropriate immonium ions are listed. They provide further information about the content of the amino acids but not about their position [1].

The strategy for the interpretation that will be discussed consists of nine components. This strategy is very effective for the interpretation of tryptic peptides obtained by electrospray ionisation. The interpretation begins with the assumption that the monoisotopic molecular weight of the peptide in its singly protonated form $(M+H)^+$ is known. Further it is useful to know about the most abundant charge state as tryptic peptides commonly contain internal histidine, lysine, or arginine residues [1].

1. Inspection of the low-mass region for immonium ions: In the first step the low-mass region is checked for the presence of immonium ions and further for the amino acid composition they are derived from.

2. Inspection of the low-mass region for b_2-ions: In the second step the low-mass region is inspected for b_2-ions and their belonging a_2-ion separated by 28 kDa.

3. Inspection of the low-mass region for y_1-ions: The next step is to find the C-terminal end of the peptide by looking for the y_1-ion.

4. Inspection of the high-mass region for y_{n-1}-ions: The fourth step is the identification of the N-terminal end by combination of the b_2-ions. Additionally the y_{n-1}-ion is identified within this step.

5. The *y*-series is extended to the lower *m/z*: With the help of the residue masses the *y*-ion series is extended backwards.

6. The *b*-series is extended to the higher *m/z*: Like the extension of the *y*-ion series the *b*-ion series is extended towards higher *m/z* by the help of the residue masses.

7. Calculation of the mass of the peptide: After the interpretation of the spectrum the mass of the peptide is calculated and the sequence is checked in agreement with the mass.

8. Reconciliation of the amino acid content with the spectrum data: In this step the content of the amino acids is checked if it matched with the observed immonium ions.

9. Identification of all ions in the spectrum: The last step is the identification of the remaining ions in the spectrum. They are checked for neutral losses (H_2O, NH_3, $HSOCH_3$), for doubly charged ions, and for any ions due to internal cleavages.

5 New Technologies and Developments in Mass Spectrometry

In the following chapter special applications and new developments using mass spectrometric techniques will be discussed. Mass spectrometry is the most promising technique not only for the analysis of proteins but for whole proteomes.

5.1 Imaging Mass Spectrometry

Imaging mass spectrometry is a new development in the direct analysis of tissue sections especially by MALDI-MS. One application area among others is the investigation of cancer tissues which makes it possible to search for many different substances in cancer cells instead of several biomarkers. It allows the differentiation between healthy and cancer tissue but also between different cancer grades. Commonly imaging MS is divided into two parts called “profiling” and “imaging”. “Profiling” means the classification of different cell types according to their protein profile based on MALDI-MS experiments and “imaging” is the distribution of each of the proteins in the different cell types [16].

The collection and the preparation of the tissue is of crucial importance as it is the basis for MS experiments. It starts with the surgical removal of the tissue of a living or dead organism. This has to be done in the right way in order to avoid any form of delocalisation or degradation of peptides and proteins. The removal from dead organisms has to be done immediately after death to avoid tissue degradation. Depending on the experiment and the tissue it might be necessary to destroy all proteolytic enzymes present in the tissue before freezing or fixation of the sample. In a next step the tissue is sectioned with the right thickness at adequate temperature (-20 °C). Before cutting the tissue has to be fixed, for example embedding in non-polymer material like 10 % gelatine or agarose. The obtained tissue sections are then commonly fixed on an Indium-Tin-Oxide conductive glass plate by warming the tissue section and the sample plate [17].

The next step in the preparation of the tissue samples is a washing step. This washing step improves signal intensity and signal quality by removing salts, lipids and haemoglobin. Washing reagents might be organic solvents such as ethanol, methanol, acetonitrile or more

aggressive agents like chloroforme or hexane. After depositing a large droplet of the solvent onto the tissue, it is recollected and the tissue is then dried again. The recollected solvent is tested for proteins to control the loss of proteins. The washing with several different solvents carries the risk of diffusion of molecules due to the removal of lipids. As different tissues behave different the washing steps have to be customized to the tissue. The integrity of the tissue is controlled by staining after the end of the measurement to ensure that the washing procedure was adequate [17].

After washing a matrix is deposited onto the tissue section. In general the matrix is composed of small organic acids, like derivatives of benzoic acid or cinnamic acid. Depending on the composition of the matrix molecules with different molecular weight are dissolved in the matrix forming matrix-analyte crystals. There exist several different procedures for the deposition of the matrix but important for all is that the spatial integrity of the sample is not destroyed. Further it is important that the obtained matrix layer is homogeneous and not too less and not too much because if the layer is too thin only a small amount of proteins will be dissolved in the matrix which causes a loss in signal intensity. If there too much matrix on the tissue this might cause a migration of the analytes. In principle there exist two methods for the deposition of the matrix: individual droplets on desired regions of the tissue or the formation of a continuous matrix layer by an aerosol spray. This can be done either manual or automatic whereby better results are obtained by automated deposition systems. The automated placement of droplets has the advantage of very uniform and precisely placed droplets but the droplets are much greater in size than the droplets from a spray. Therefore the spatial resolution with the droplet method is limited. Better resolution is obtained using a spray system as the obtained matrix crystals are much smaller which increases the spatial resolution. However, the evaporation of the solvent is much higher using spray systems which limits the time for the analytes to leave the tissue. Therefore with spray systems the temperature and the humidity of the environment have to be controlled strictly. Another method for matrix deposition would be the sublimation of the matrix. No additional solvent is used which increases the purity of the matrix and increases the resolution [17].

After all these preparation steps a MALDI MS is done described already in a previous chapter. In former days clinical application was limited as a analysis of a small tissue micro array would last seven days and produces around 50 GB of data. Nowadays by increased throughput such analysis can be done within 1.5 days [18]. If not enough information can be

obtained from the mass spectrometric experiment (protein identification) it is possible to introduce a step of tryptic digest in the tissue after the washing. This will increase the information out of the MS experiment [17].

5.2 Analysis of PTMs by Mass Spectrometry

Post-translational modifications (PTMs) are of great interest in the analysis of proteins. As they are not coded in the genome they are not predictable. PTMs change the properties of a protein, such as localisation, function and lifetime due to changes in solubility, pH value or stability. Most common PTMs are phosphorylation and glycosylation, but also acetylation, nitrosylation or deamidation [19], [20] might occur. The changes in mass and in *m/z* due to the PTMs allow their detection, which is important for studying the influence of PTMs on diseases like cancer, heart diseases or neurological diseases. The probability of an amino acid to be modified depends on character of the side chain. Aliphatic (alanine, valine, leucine and isoleucine) and aromatic (phenylalanine) amino acids are not expected to become modified due to a lack of reactivity of the side chain. On the contrary, side chains containing amine groups, carboxylic groups or thiol groups might be modified in various ways [1]. Nevertheless, several difficulties have to be overcome analysing PTMs by tandem mass spectrometry. One critical aspect might be the mass accuracy due to similarities of several modifications. Mass accuracy is rather achieved in lower molecular mass ranges which makes a digest producing smaller peptides suitable for analysis. Further problems are on the one hand the low stoichiometry of the post-translational modifications which requires an enrichment step before mass spectrometric experiments. On the other hand acidic modifications such as phosphorylation and glycosylation lower the ionic yield and lead to a suppressed signal. If these limitations can be overcome tandem mass spectrometry is a powerful tool in analysing PTMs in proteins [1], [19]. As phophorylation and glycosylation are the most common post-translational modifications some strategies for their detection will be given.

Phosphorylation is a covalent, reverse attachment of a phosphate group to the side chain of a serine, threonine or a tyrosine. Almost 50 % of all proteins are phosphorylated and it is estimated that over 100,000 phosphorylation sites are present in the human proteome. It is documented that various forms of cancer or Alzheimers disease are connected to different

states of phosphorylation. As there are several phosphorylation sites in a protein it is necessary to identify the single sites of modification and not only the global phosphorylation state. The number of isoforms create a complex system which makes the analysis of whole proteomes to a major task. The low abundance and the suppression effect make an effective enrichment and a simplification due to separation necessary. Traditionally a two-dimensional gel-electrophoresis was carried out combined with specific labelling (radioactive or phopshorspecific stains) or Edman degradation. Yet these methods have been time and labour consuming and a rather huge amount of sample is required to come up with satisfactory results. Nowadays phosphorylation can be easily determined by tandem MS as this an increase in mass of +80 Da. The mass increase is observed when the phosphorylation is stable (tyrosine). If it is less stable (serine, threonine) in CID activation a loss of phosphoric acid is observed (-98 Da) and the remaining serine and threonine have a reduced mass of -18 Da due to a loss of water from their original structure. As already mentioned before one strategy for simplification is the isolation and enrichment of phosphopeptides by a chromatographic step before MS analysis. This reduces the complexity of the sample and therefore facilitates the often manual interpretation of the obtained MS data. Chromatographic separation is commonly used today: immobilized metal affinity chromatography, metal oxide affinity chromatography and ion-exchange chromatography [19].

The probably most abundant form of post-translational modification is the glycosylation of proteins. It is estimated that more than 50 % of all proteins are glycosylated. Glycosylation also has several influences on the structure, the lifetime, the folding and the aggregation of proteins and is therefore connected to several diseases. Glycosylation is much more complex than phosphorylation due to the variety of glycans that can be attached. In principle two different forms of glycosylation are distinguished: the *N*-glycosylation to an asparagine residue and the *O*-glycosylation to a serine or threonine side chain. Best results of the analysis of complex mixtures are nowadays obtained by ESI-MS that is directly coupled to a separation device. With this arrangement molecular masses of the modified proteins are received but not the exact composition of the glycans. For more information the released glycans (chemically or by an enzyme) are analysed by MS after a appropriate separation step. For gaining more information also a digest of the protein might be included. The affinity of glycoproteins for lectins is exploited for the enrichment: lectin affinity chromatography. Glycopeptides might be enriched by size-exclusion chromatography, hydrophilic interaction

chromatography (HILIC) and methods based on graphitized-carbon material additionally to the lectin methods [19].

5.3 Future Perspectives of Mass Spectrometry in Proteomics

The probably greatest challenge in MS of proteins is the quantitative analysis of whole proteomes. Basically it can be differentiated between isotope labelling or labelling with specific mass tags and label-free techniques. The adding of mass tags can be achieved either metabolically, enzymatically, by chemical means or by providing of spiked peptide standards. Using label-free methods two methods with different strategies are utilized: one is based on the normalized intensities of the peptide signals and the other is the so-called “spectral counting” which means that the more protein is in the sample the more fragment spectra for peptides derived from the protein can be collected [21], [22].

The method of labelling is based on the principle that the mass difference between the unlabelled and the labelled peptide is detected and the quantification is then achieved by comparing their signal intensities. It is assumed that both forms behave identically and no discrimination is observed. The easiest method is the metabolic introduction. In this case the cell cultures are grown either on ^{15}N-enriched cell culture medium or the culture medium contains $^{13}C_6$-arginine or $^{13}C_6$-lysine. The advantage of arginine or lysine after tryptic digests is that every peptide contains one tag. Another advantage is that treated and untreated cell lines can be combined at the level of intact cells and undergo then the same treatment. If errors occur every protein population undergoes the same error. A problem of this technique is that it is very work intensive which means that is not practical and in many cases often not possible because not every cell line can be grown on synthetic media [21], [22].

The chemical or enzymatic modification is generally done in vitro. The enzymatic introduction can be either done during digestion or afterwards in a separated step. A very simple way is the introduction of ^{18}O. The advantage of enzymatic labelling over chemical labelling is that in enzymatic labelling side reactions are avoided. For chemical treatment every reactive side chain can be used. Commonly the side chains of lysine and cysteine are modified. The reagent that is used contains not only the mass tag but also for example a biotin group for washing and enrichment steps. Reagents that modify cysteine are used for the

investigation of complex samples as cysteine is a very rare amino acid. For chemical labelling many other methods exist, such as modification of the N-terminal amino acid or the modification of the carboxylic acid in side chains or C-terminal amino acid [21], [22].

Another method of quantification with labelling is the use of a labelled standard. The probably easiest way is to add a labelled standard of a protein with a known concentration to a digest. This is useful for the quantification of one or only several proteins. This method is also known as AQUA – absolute quantification of proteins. One limit is that have to make a "guess" what the concentration of the protein will be to be in an appropriate level of measuring. Another problem would be the presence of isobaric peptides (peptides with same molecular mass). This problem can be overcome with a combination of LC (comparison of retention times) and specific fragment ions in tandem MS experiments. If there are losses or enrichment in the sample preparation it is possible that AQUA experiments do not reflect the right concentration expressed in the cell [21], [22].

The normalized peptide method, which is one technique of the label-free methods, is based on the extraction of one *m/z* of a peptide of every LC-MS/MS run and the peak is then integrated over the whole run. Then the intensity of one peptide is compared to the same *m/z* in other experiments. As a result relative quantitative information is obtained [21], [22].

As already mentioned a second method in label-free quantification is the counting of fragment spectra derived from one protein. It is assumed that the more protein is in the sample to more fragment spectra are observed. This method also generates also relative quantitative information because the number of obtained spectra is then compared with results from other experiments. For quantification with reliable results several fragment spectra of one protein should be observed because chemical behaviour of proteins differs and therefore the linearity range of proteins is not the same [21], [22].

It can be concluded that label-free method are not that accurate then methods using labelling techniques but nevertheless they might be a good choice for several reasons. In general it can be assumed that the number of preparation steps in every experiment should be as low as possible and the label-free methods do not need any labelling step. Besides the saving of labour also no chemicals for labelling are required. Further in principle the number of experiments that can be compared is unlimited. These arguments allow label-free methods to be a good alternative [21], [22].

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