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The Use of Proteomics as a Novel Tool in Aflatoxin Research

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1. Introduction

The field of system biology among the disciplines, genomics, transcriptomics, proteomics and other “omics” technologies, has become one of the important scientific fields nowadays and will play a major role in investigating biological processes on a global level. Huge financial, technological and organisation efforts are needed to perform system biology based approaches. Progresses in molecular biology have led to the sequencing of the whole genome of several organisms, ranging from bacteria to various mammals including human. While the term “genomics” has already been used for a long time, new technologies such as transcriptomics, proteomics and metabolomics have been introduced during the last two decades and are beginning to expand rapidly. In general the “omics technologies” deal with hundreds or thousands of genes and/or their products such as mRNA, proteins or metabolites. Genomics is the study of genes, their variation and function by sequencing and mapping them. Similarly, transcriptomics deals with information at the messenger RNA (mRNA) level in an organism, tissue or cells at a given time by providing quantitative or semi-quantitative data. The transcriptome is however, not a straight copy of the genome, since the sequence of RNA molecules can be altered due to differential splicing and RNA editing. In contrast to the genome, which is static, the transcriptome is changing depending on environmental signals. Although, genomics and transcriptomics provide huge amount of information for understanding biological processes, the knowledge regarding the products of genes and transcripts remained underutilized. While, in last three decades predominantly genes and gene expression profiles were investigated and genomics has become the major side in biosciences, the analysis of proteins in a global approach was neglected mostly. However, since the 1990s also proteomics has gained increasing interest in the field of biosciences. The term proteomics was mentioned for the first time in Siena/Italy by Marc Wilkins in 1994 on occasion of the symposium on “2D Electrophoresis: from protein maps to genomes” (Wilkins 1997). Proteomics is defined as the qualitative and quantitative comparison of proteins in a cell or organism under defined conditions at a given time point. In other words proteomics is a term coined to comprise a field that attempts to understand the expression, function and regulation of the entire set of proteins encoded by an organism (Liebler 2002). In addition, since the proteins within a cell are the functioning units, their expression is strongly influenced by the environmental signals such as drugs, toxins, stress, age and other surrounding conditions. It is a complementary technology to genomic as well as transcriptomic research (Wilkins et al., 2007). Proteomics aims to deliver statistically

significant quantitative as well as qualitative data, which have to be validated by different methods like western blotting and additionally verified using transcriptomics data. Different strategies have been used within this new discipline such as analyzing the whole proteome, determining the interactome, investigating the secretome and verifying glycoproteome as well as the phosphoproteome etc.

While Protein chemistry has long tradition, proteomics can be seen as the “child” of this discipline. However, there are major differences between traditional protein chemistry and its “child” proteomics: While protein chemistry is interested in only one or a set of proteins, its function and structure, proteomics deal with hundreds of proteins in complex mixtures and their discovery by database matching. Identification and quantification of proteins in a complex mixture is an analytical challenge. If comparing proteomics with genomics and transcriptomics fundamental discrepancy from methodological point of view can be distinguished. DNA and mRNA are physico-chemically homogenous molecules, whereas the researchers in case of proteins are confronted with a different situation. In the case of proteins neither amplification nor hybridization is possible. The proteins exhibit a fully different chemical behaviour like hydrophobicity and hydrophilicity as well as completely different concentration ranges of low and high abundant proteins ranging from millimol to atomol. Additionally, posttranslational modifications, proteolytic degradation and protein turnover lead to a much complex situation in the analysis of proteome compared to genome or transcriptome. Therefore selective and sensitive detection methods and a combination of different techniques are necessary to be able to perform reliable proteome analysis. As a consequence, the cost of instruments and the use of various methods are much higher. Another complementary OMICS technology is metabolomics, which deals with the separation, identification and quantitative determination of thousands of metabolites in biological samples. As a consequence the combination of all systems biology based data will lead to knowledge about the functional biology of whole-organism (Humphery-Smith & Hecker 2006). As other scientific fields proteomics has also found broad interest by mycotoxicologists and fungal biologist. Thus, in the last decades the field of fungal proteomics and related areas have been expanding rapidly (Fig. 1).

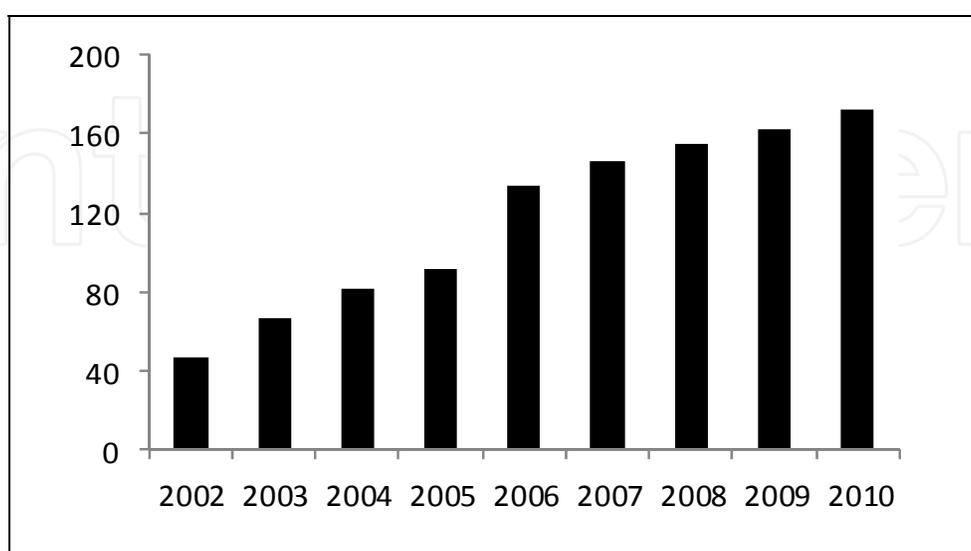


Fig. 1. The number of new articles (including reviews) that have appeared in each of the past eight years related to fungal proteomics

2. Methods in proteome analysis

The early development of proteomics goes back to the introduction of two dimensional gel electrophoresis (2-DE). The current methods of choice in proteomics are one- or two-dimensional polyacrylamide gel electrophoresis (1-DE or 2-DE) followed by nanoHPLC combined with electrospray mass spectrometry (LC-ESI-MS) or matrix-assisted laser-desorption ionization / time of flight mass spectrometry (MALDI-TOF). Accordingly, two strategies for the analysis of proteome can be distinguished, namely “gel-based” and “gel-free” proteomics. In “gel-based” proteomics polyacrylamide gel electrophoresis (PAGE) in either one or two dimensions are used. (Fig.2). Mainly 2-DE in combination with either MALDI-TOF-TOF or nanoHPLC-MS-MS is applied. Due to high resolution power of 2-DE hundreds of proteins can be separated in a single gel. Additionally, 1-DE in combination with nanoHPLC-MS/MS is frequently used in proteomics (Chandramouli & Qian 2009).

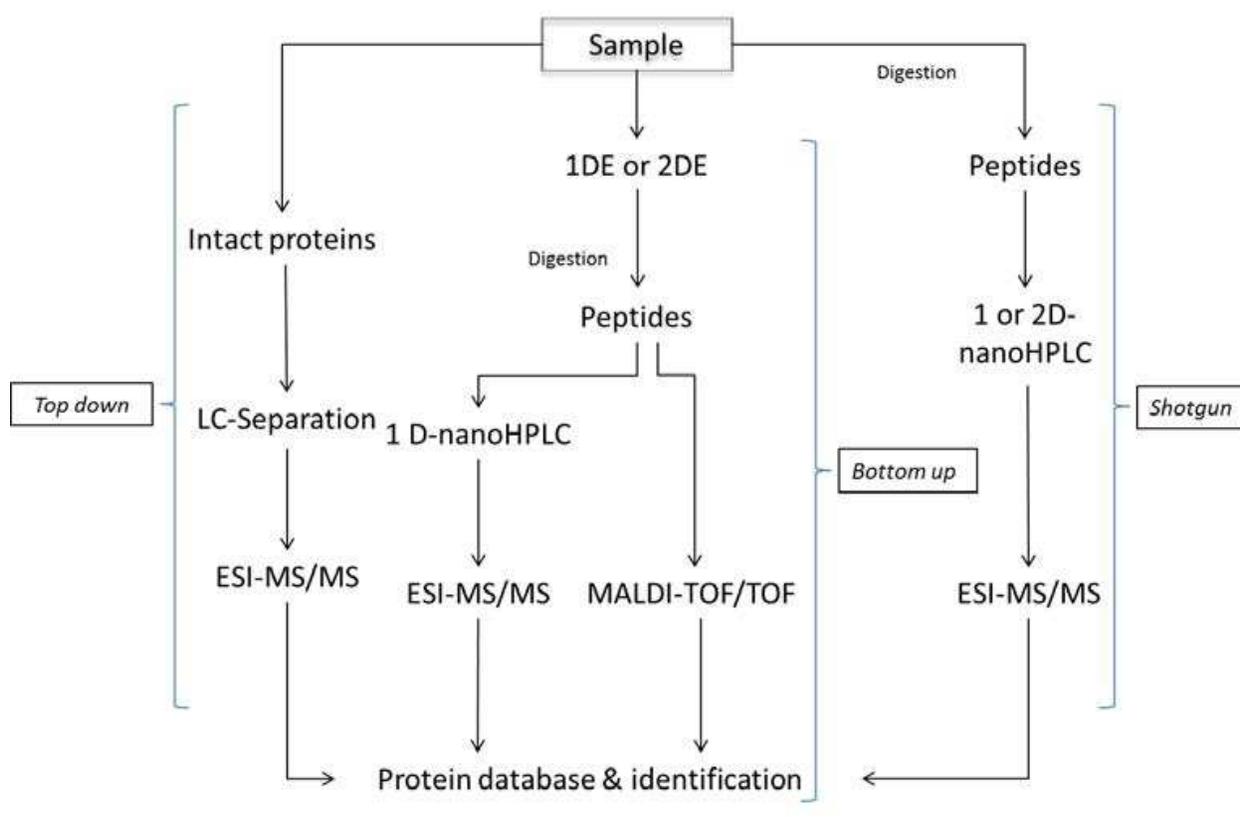


Fig. 2. Schematic illustration of strategies used in proteome analysis

In contrast to the “gel-based” approach, the “gel-free” approach uses chromatographic separation of internal peptides derived from digested proteins. Ion-exchange (IE) and reverse-phase liquid chromatography (RP-HPLC) are used in two dimensional approaches to achieve high resolution separation of peptides (2D-LC), which is also a versatile and powerful tool. However, a “gel-based” proteomic approach is much cheaper than a “gel-free” one if taking the instrument cost into consideration.

Despite the separation performance, the first step in a proteomics approach is the sample preparation, which is extremely crucial since the results are deeply influenced by this first step.

2.1 Sample preparation strategies in fungal proteomics

The aim of the sample preparation in proteomics is the effective extraction of all expressed proteins from an organism or tissue with the possible highest efficiency, by solubilizing them. Without appropriate extraction and solubilisation, further separation and analysis of proteins is definitely not possible and the proteomics approach will fail (Posch 2008). Especially in fungal proteomics, to our experiences, the appropriate sample preparation plays a key role in protein extraction from the cell. Filamentous fungi contain a rigid cell wall and the extraction of intracellular proteins is therefore difficult. The cell wall is involved in many important functions, such as physical protection, osmotic stability, selective permeability barrier, immobilized enzyme transport, cell to cell interactions, and morphogenesis. Additionally, the cell wall is involved in virulence, pathogenicity, antigenicity, immunomodulation and adhesion to host substrate in pathogen fungi (Chaffin et al., 1998). Most of the studies were reported on the cell wall of *Saccharomyces cerevisiae* (De Groot et al., 2005; Klis et al., 2006; Ruiz-Herrera et al., 2006), which is generally composed of glucans (with β -1,3 and β -1,6 linkage), chitin (N-acetylglucosamine polymers), and proteins. These proteins are often highly O- and/or N-mannosylated leading to an elevated complexity (Pitarch et al., 2008). Generally, the cell wall proteins are very difficult to analyze due to their low solubility, hydrophobic nature and low quantity.

Cell lysis is necessary in order to extract the intracellular proteins from filamentous fungi. There are numerous cell lysis protocols used for extraction of proteins, which include: grinding with liquid nitrogen using mortar and pestle (Hernandez-Macedo et al., 2002; Grinyer et al., 2005; Shimizu & Wariishi 2005; Shimizu et al., 2005; Fernandez-Acero et al., 2006; Kniemeyer et al., 2006; Yajima & Kav 2006), mechanical grinding using glass beads (Nandakumar & Marten 2002), chemical lysis (Riezman et al., 1983), and enzymatic lysis (Conzelmann et al., 1988). In a review by Nandakumar & Marten, different lysis methods were compared to extract intracellular proteins of *A. oryzae* for 1-DE and 2-DE (Nandakumar & Marten 2002). The authors tested four lysis cell protocols: (i) boiling in strong alkali, (ii) boiling in sodium dodecyl sulfate (SDS), (iii) chemical lysis in Y-PER[®] reagent, and (iv) mechanical lysis via rapid agitation with glass beads in a Mini-BeadBeater[®]. The authors reported that the “mechanical lysis via rapid agitation with glass beads” method seems to be most suitable for the protein extraction and showed good patterns on 1-DE and 2-DE gel electrophoresis.

Most of the protein extraction methods and lysis protocols applied to fungal proteomics were overtaken from plant proteomics with some modifications. Mainly detergents like SDS or 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) as well as chaotropic agents (urea and thiourea) in combination with a reducing agents such as DTT are used. In order to prevent proteolytic degradation of proteins and thus, changes in the proteome pattern, protease activity has to be inhibited. Consequently it is necessary to add protease inhibitors to the lysis buffer. In general a combination of different protease inhibitors in form of a cocktail is recommended. Furthermore, the addition of carrier ampholytes improves the solubility of proteins (Westermeier et al., 2008).

Major proteins of *Aspergillus ochraceus* were extracted with the help of a mortar and pestle in liquid nitrogen and were separated on 1-DE as well as 2-DE (Rizwan et al., 2010). Similarly, intracellular proteins from *A. fumigatus* were extracted by mortar and pestle using liquid nitrogen followed by brief sonication (Carberry et al., 2006).

2.1.1 Protein precipitation

During the cell lysis other interfering substances like phospholipids and nucleic acids can be co-extracted and will be visualized in the acidic part of the gel (Westermeier et al., 2008).

Therefore the crude extract should be purified prior to 2-DE. A very common strategy is to perform precipitation to remove the contaminants after cell lysis. Proteins can be easily and effectively precipitated by ammonium sulfate, chloroform/methanol, trichloroacetic acid (TCA) etc. In the case of TCA precipitation, TCA has to be removed by washing the pellets with acetone in order to prevent problems with isoelectric focusing (IEF). Sodium hydroxide treatment for short time resulted in improved solubilization as studied by (Nandakumar et al., 2003). Another advantage of precipitation is that other interfering substances like detergents and salts can be removed as well (Lopez 2007). In addition, precipitation leads to irreversible inactivation of proteases (Westermeier et al., 2008). Following the precipitation the proteins have to be again dissolved by adding chaotropic agents like urea and thiourea as well as detergents. Especially the addition of zwitterionic detergents like CHAPS were reported to facilitate the protein resolubilization (Kniemeyer et al., 2006). Mechin et al., 2003 reported the most appropriate resolubilization mixture should consist of two detergents (CHAPS and SB3-10), two chaotropes (urea and thiourea), two reducing agents dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) and two types of carrier ampholytes (pH 4-6.5 and pH 3-10 ranges). Despite advantages of precipitation in removing interfering substances, the procedure of precipitation and resolubilization rarely delivers 100% recovery of all proteins resulting into changes in protein pattern (Lopez 2007).

2.2 Two-dimensional gel electrophoresis in proteome expression analysis

Decades after introducing 2-DE, this technique is still the most important and basic research tool used in protein chemistry. Moreover, the establishment of this technique contributed significantly in the development and establishment of proteomics. 2-DE was first introduced independently by (Klose, 1975) and (O'Farrell, 1975). Thereby proteins are separated based on their isoelectric points (pI) in the first dimension and on their molecular masses in the second dimension. Proteins have zwitterionic character due to their acidic and basic groups resulting in different charges depending on the pH of their environment. The pI is the pH at which the net charge of the protein is zero and no migration of the molecules in an electric field is possible. The technique of separating proteins by their pI is called isoelectric focusing (IEF). There are two possibilities to perform IEF, either by using carrier ampholytes generated pH gradients or applying immobilized pH gradient (IPG) (Smith 2009). While in the original method carrier ampholytes in thin gel rods in glass or plastic tubes were used, nowadays mainly IPG strips are used in the first dimension. IPGs consist of a pH gradient in gels prepared by co-polymerization of acrylamide monomers with acrylamide derivatives containing carboxylic groups (Lopez 2007). In comparison with the carrier ampholytes based method, the IPG strips are more stable, highly reproducible and allow the focusing of acidic as well as basic proteins (Görg & Weiss 2000; Westermeier & Barnes 2004). Furthermore, IPGs exhibit a large separation capacity and no gradient drift. Meanwhile IPGs in different type of pH gradients and strip lengths are commercially available. The use of narrow-range gradients (pH 3-6, 4-7, 5-8, and 7-10) in various lengths (7, 11, 17, 18, and 24 cm) will improve the resolution of protein separation. The prepared protein extracts can be then loaded by *in-gel* rehydration of IPGs or by cup loading procedure. Once the sample is loaded on the strips, the electric field is applied at 8000 or 10,000 V in a dedicated apparatus with an effective cooling system at 20°C temperature.

Following equilibrating with the anionic detergent SDS, the strips are loaded onto a SDS-PAGE apparatus. SDS denatures and binds to the proteins building a complex with a net negative charge on each protein. The basic principle of the separation is that negatively

charged proteins will migrate through the polyacrylamide toward the anode upon the application of an electric field. By applying electric current the proteins are separated based on their size or Stokes radii (Laemmli 1970). Dependent on the percentage of polyacrylamide the logarithm of the molecular weight is a linear function of the distance of migration of the proteins. Thus, by using known marker proteins the molecular mass of the proteins can be estimated. Mostly a homogeneous gel is used in second dimension since the proteins are separated in a first dimension (Westermeier et al., 2008). Fig. 3 shows a 2-D GE separation of whole protein extract from *A. ochraceus* NRRL 5175. The protein extract (350 μ g) was subjected to IEF followed by SDS-PAGE and Coomassie blue staining. Numbers refer to protein identifications. As can be seen in Fig. 3 on the y-axis of the gel the molecular weight of the proteins appear and on the x-axis the pH gradient (pH 4-10).

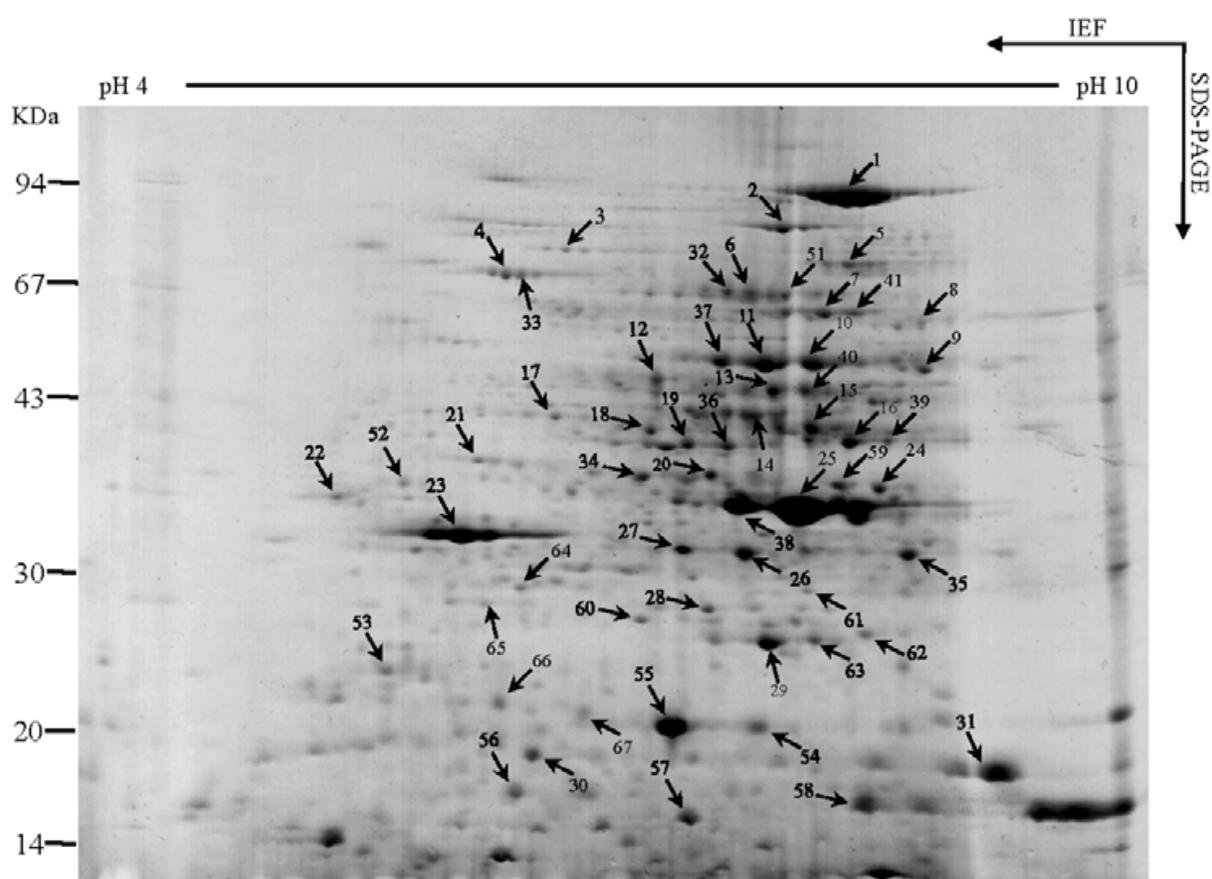


Fig. 3. Two dimensional Gel of the whole protein extract from *A. ochraceus* (Rizwan, 2010)

After the electrophoretic separation, the proteins can be detected by different staining methods that exhibit various sensitivities. There are many dyes commercially available like coomassie blue, colloidal coomassie blue and silver nitrate with sensitivities of 50 ng protein/spot, 10 ng protein/spot and 0.5 ng protein/spot respectively (Chevalier 2010). Additionally, there are fluorescent dyes such as Deep Purple, Sypro Ruby etc., which show sensitivities of about 1 ng protein/spot. In the case of fluorescent dyes an appropriate fluorescent scanner has to be used to visualize the spots. MS-compatibility of silver staining is reached by doing the staining without glutaraldehyde.

The introduction of the "Difference in Gel Electrophoresis (2D-DIGE)" technique (GE Healthcare) was a major progress in semi quantitative gel based proteomics (Ünlü et al., 1997). DIGE is a pre-electrophoretic multiplexed fluorescent staining method. Chemically modified cyanine dyes (CyDye) that have different excitation and emission wavelengths are used. The major advantage of 2D-DIGE is the ability to reduce, to a large extent, gel to gel variation. This is due to the fact that proteins of different proteome states (samples) migrate in the same gel and under same electrophoretic conditions. There are three different dyes Cy2, Cy3 and Cy5, which label the proteins by covalently binding to the lysine residues (minimal labeling). In minimal labeling only 400 pmol/L of dye is added to 50 µg of protein, which means that only 3 to 5% of the proteins will be labeled and 95-97% proteins remain unlabeled (Westermeier et al., 2008).

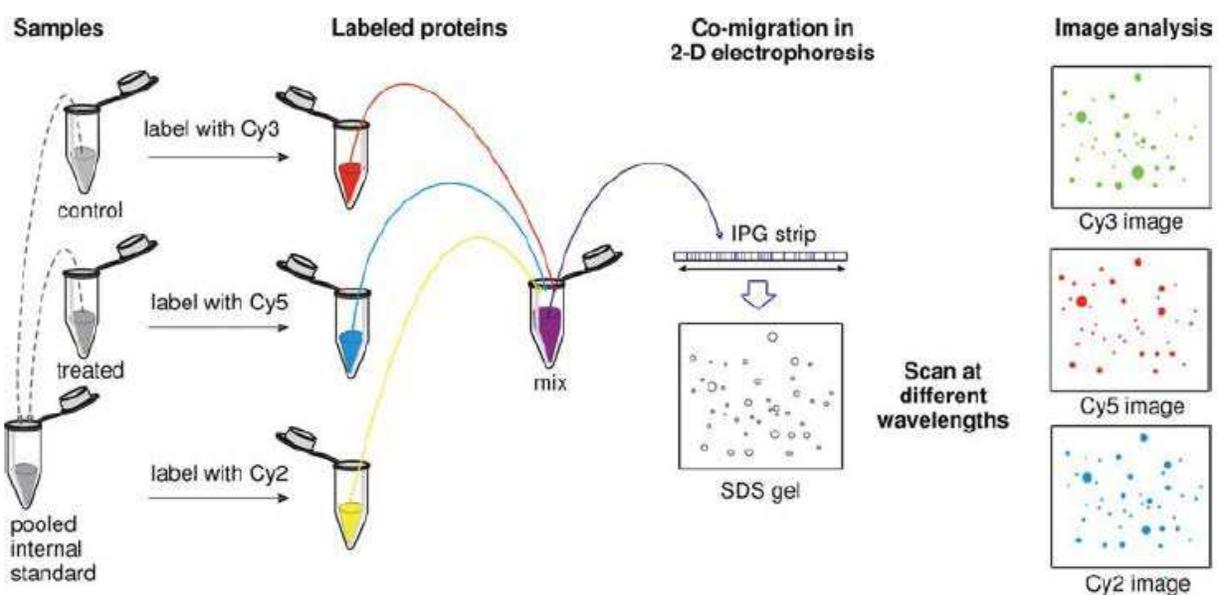


Fig. 4. The principle of 2D-DIGE from (Westermeier et al., 2008).

Mostly two samples (control and treated) in an experiment are labeled by Cy3 and Cy5. Additionally, aliquots of each sample are pooled and then labeled by Cy2 as internal standard (Fig 4, 5). In contrast to lysine minimal labeling, saturating labeling of cysteine residues by Cy3 and Cy5 can be performed, which is very sensitive (Sitek et al., 2005; Westermeier et al., 2008). After electrophoretic separation the gel can be visualized by a fluorescent scanner by exciting each of the dyes with a different wavelength. By using an appropriate software it is possible to obtain semi-quantitative comparison of different proteome patterns within the same gel. Another advantage of 2D-DIGE is its wide linear dynamic range and the possibility to visualize the spots by silver or Coomassie blue staining afterwards.

A drawback is however, that only two different fluorescent reagents are commercially offered for "complete DIGE" and the costs of the reagents are rather high-priced for larger proteomics projects. Furthermore, limitations in loading capacity, quantitative reproducibility, difficulties in handling, and interfacing problems to mass spectrometry limit the analysis depth and comprehensiveness of the gel-based proteomics studies.

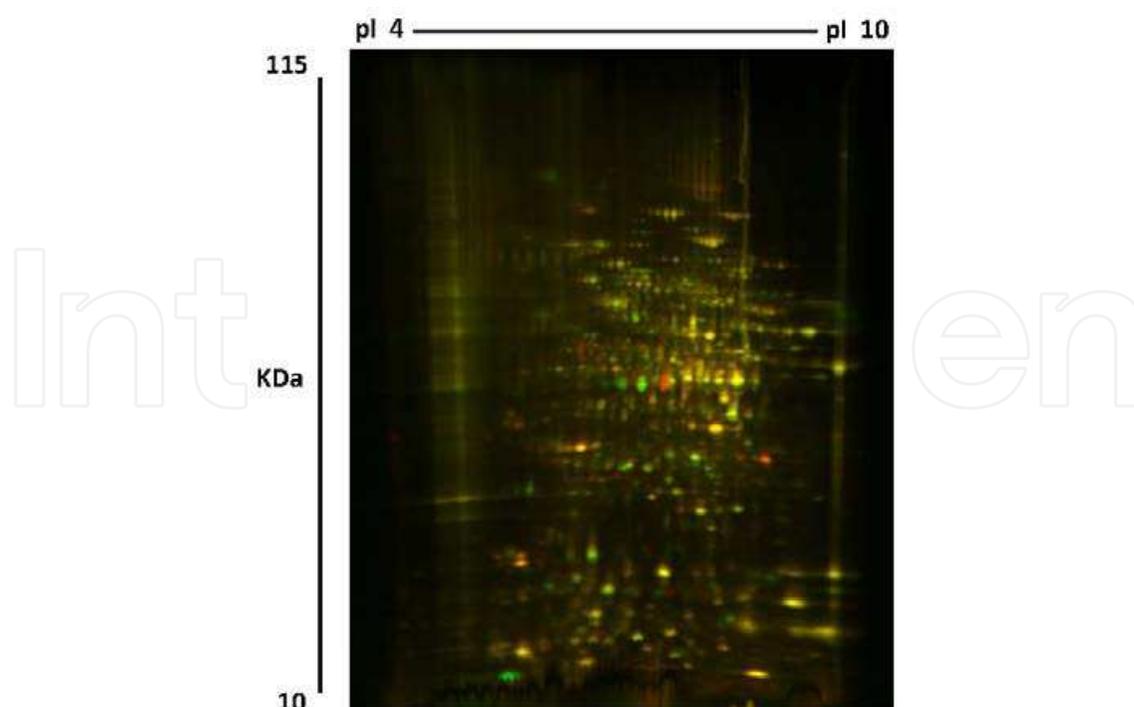


Fig. 5. 2D-DIGE of proteins from *A. ochraceus* NRRL 5221 cultivated in malt extract (ME) and yeast extract (YES) broth. Green (Cy3): 5221 cultivated in ME, and red (Cy5): 5221 cultivated in YES (25 μ g of protein extract was loaded). Yellow represent those proteins expressed in both cultures (Performed by Ingrid Miller, with friendly permission), (Rizwan, 2010)

Overall, 2-DE was and is still the method with the highest resolution for separating complex mixture of proteins. One of the advantages of 2-DE is the possibility to extract the separated proteins and/or its internal peptides from the gel for MS-based identification. For biochemical characterization of proteins after 2-DE it is possible to detect them by performing western blotting. Beside the high separation capacity large amount of protein can be loaded onto the gel.

In fact, the introduction of IPGs has tremendously facilitated the 2-DE technique. On the basis of IPGs and their commercial availability, 2-DE became more user friendly. In addition, sample loading capacity in 2-DE is satisfactory, however not unlimited. As an example the loading capacity of narrow-range IPGs is much higher than those of broad-range IPGs (Chevalier 2010). Nevertheless, 2-DE shows limitations and advantages like any other method. The detection of low-abundance proteins as well as high molecular weight proteins are major problems in 2-DE. Another drawback of 2-DE is the weakness of this technique when separating very alkaline proteins like ribosomal proteins or histones (Lopez 2007; Chevalier 2010). Additionally, there are also some innate limitations in reproducibility and dynamic range as well as the limitation of detecting post-translational modifications.

Especially in the case of membrane proteins 2-DE has difficulties to resolve and separate proteins due to their hydrophobicity. Their poor solubility leads to “smearing” effects within the IPG strips (Liebler 2002). In these cases a different 2-DE strategy was performed by using 16-benzyltrimethyl-n-hexadecylammonium chloride (16-BAC) a cationic detergent in the first dimension followed by a SDS-PAGE separation in the second dimension (Zahedi et al., 2007).

Additionally, gel-free proteomic methods, such as the multidimensional protein identification technology are also becoming popular in proteomics. However, there is up to now no technology that matches 2-DE in its ability for routine parallel expression profiling of large sets of complex protein mixtures. 2-DE delivers a map of intact proteins, which reflects changes in protein expression level, isoforms, or post-translational modifications. Therefore 2-DE using IPGs combined with protein identification by mass spectrometry is at present the workhorse for the majority of current proteome projects.

2.3 Mass spectrometry

Mass spectrometry has revolutionized the analysis of biomolecules from the beginning of the 1990s. Without mass spectrometry (MS) the identification and characterization of proteins, glycans and many other biomolecules in a global approach would not be possible. The mass spectrometer consists of an interface, an ion source, mass analyzer and detector. After ionization of the molecules in the source the ions are separated based on their mass to charge ratio (m/z) in the mass analyzer and will be subsequently detected. The so called gas phase ions are accelerated in a field-free space, magnetic or electromagnetic fields towards the detector. Different mass analyzers such as time of flight (TOF), magnetic sectors, quadrupoles, quadrupole ion traps and linear ion traps are commonly used (Wilkins et al., 2007; Faull et al., 2008). Other high resolution mass analyzers such as orbitrap and Fourier transform ion-cyclotron resonance (FTICR) are also used in proteomic studies. Hybrid instruments such as a combination of quadrupole/quadrupole and quadrupole/TOF or linear ion trap/orbitrap analyzer are applied routinely (Fig. 6).

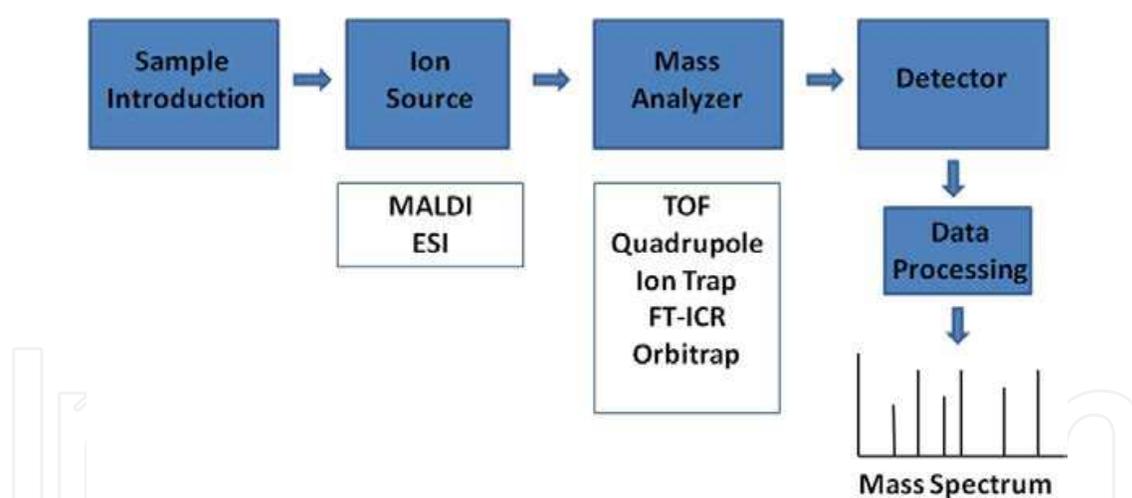


Fig. 6. Block diagram of a mass spectrometer in proteomic approach (modified from (Faull et al., 2008))

Currently, the most commonly used ionization techniques for protein identification are MALDI and ESI in combination with tandem mass spectrometry (MS/MS). These are soft ionization methods which allow ionization of biomolecules without destroying them. The development of desorption ionization methods such as MALDI and the introduction of ESI in combination with HPLC have promoted mass spectrometry to an essential tool in proteomics (Karas et al., 1987; Karas 1988; Hillenkamp et al., 1990). With the help of the so called "biological mass spectrometry" it is possible not only to identify the proteins but also to determine the amino acid sequence and characterize the post-translational modifications

of proteins. The development of both soft ionization methods was honored with the Nobel Prize for chemistry in the year 2002.

2.3.1 Matrix Assisted Laser Desorption Ionization (MALDI)

In MALDI, gas-phase ions are produced from large, non-volatile and thermally unstable compounds such as proteins. The ionization of biomolecules is facilitated by using appropriate matrices. The sample is mixed with these mostly low molecular weight compounds, for example α -cyano-4-hydroxy cinnamic acid or 2,5-dihydroxybenzoic acid, which have a strong absorption at the laser wavelength. The matrix along with the sample is then spotted on a target plate or mixed directly on the target. The matrix imparts a key role by strongly absorbing the laser light energy and causing, indirectly, the vaporization of the analyte when the laser pulses of known wavelength hit the crystals inside the source of the mass spectrometer (Fig. 7). The matrix also serves as a proton donor in positive ionization mode and receptor in negative ionization mode to ionize the analyte (Hillenkamp & Peter-Katalini 2007). The ionization depends critically on the matrix-analyte combination, not on the number of acidic or basic groups of the analyte (Beavis et al., 1990). The ions produced are then accelerated towards the analyzer by applying an electric field. In most cases time of flight (TOF) is used as the mass analyzer for MALDI.

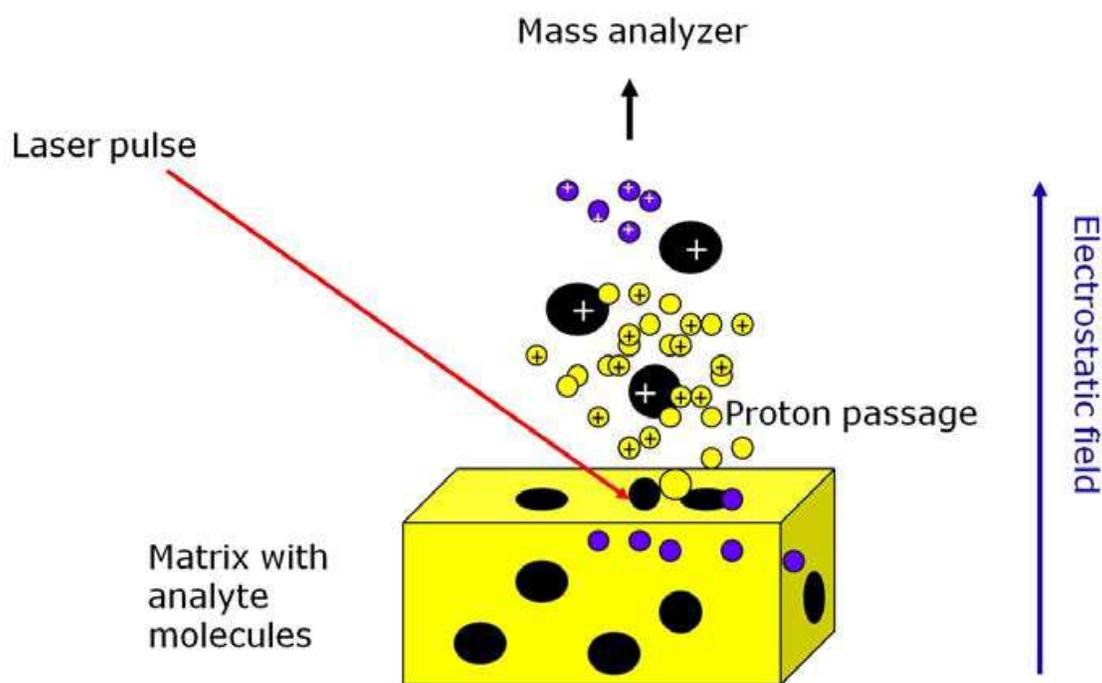


Fig. 7. Schematic illustration of the MALDI ionization. Modified from (Lottspeich & Zorbas 2006)

2.3.2 Electrospray Ionization (ESI)

The basic principle of ESI involves the introduction of a continuous stream of liquid through a capillary to the ion source at atmospheric pressure (Cech & Enke 2001). An electric field is obtained by applying a voltage between the capillary tip and the counter-electrode, i.e. the mass spectrometer entrance. Droplets are generated and vaporized continuously towards the entrance. Droplet fission will occur when the repulsion between the charges on the

surface becomes too high, producing new smaller droplets, as shown in Fig. 8 (Wilkins et al., 2007; Westermeier et al., 2008). The ESI interface can be coupled to HPLC, capillary or nano-HPLC (flow rates of 20 nl/min-200 μ l/min) as well as to capillary electrophoresis. Generally, multiply charged molecular ions are generated in ESI reducing the mass of proteins of interest. Therefore large proteins can also be analyzed by ESI in mass spectrometers with limited dynamic range.

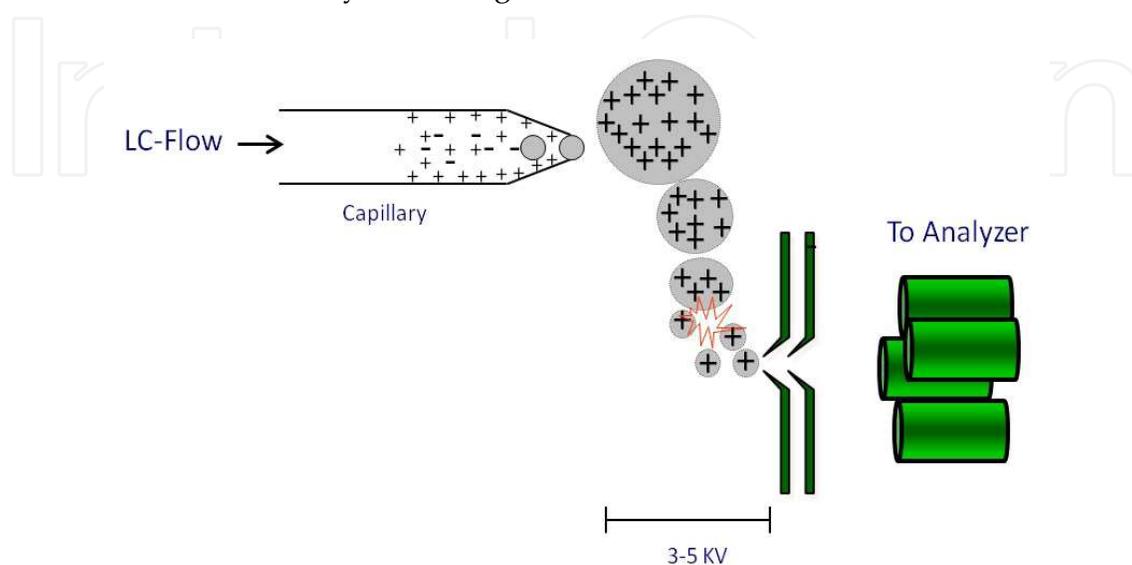


Fig. 8. Principle of ionization in ESI interface

In the last decade, nano electrospray has become the method of choice, when performing HPLC-MS/MS for separating internal peptides and identifying them. Nano electrospray was first introduced by (Wilm & Mann, 1996) and has found its main application field in proteomics. The use of nano flow has several advantages over the conventional LC. The sensitivity is much higher since the column I.D. and flow rate are reduced to 20–100 μ m and 50–600 nl/min respectively. Typically 300 nl/min for a 75 μ m I.D. column are used in separation of peptides. Additionally the nano flow rate is highly compatible to MS since better ionization efficiency is achieved with reduced flows resulting in an improvement of mass spectrometric sensitivity. In contrast to 2-DE, the on-line nano-HPLC-MS can be easily automated. Recently, using dedicated nano ultra-performance LC (UPLC), columns with smaller particles (1–3 μ m) can be used by pressures around 700 bar. Nowadays, the use of the reversed-phase nanoHPLC-ESI-MS/MS has become state of the art for the separation of complex peptide mixtures and identification of unknown proteins. Performing nano-HPLC, an appropriate nano spray source has to be exploited.

If complex protein mixtures (shotgun proteomics) have to be analyzed by nano-HPLC-MS/MS, a better peak resolution in HPLC needs to be achieved. In this case the combination of several chromatographic techniques is necessary. Two-dimensional nanoHPLC is performed using strong cation exchange chromatography in the first dimension and a reversed-phase chromatography in the second dimension (2D-LC). This separation strategy of peptides has become the key component of “gel free” proteomics, which is also called Multidimensional Protein Identification Technology (MudPIT). Nevertheless, 2D-LC is a highly sophisticated technique, which needs well trained human resources. For complex peptide mixtures the number of protein identifications can be increased by approximately 25%, when using two dimensional nano-HPLC (Westermeier et al., 2008).

2.3.3 Identification of proteins

There are three different MS based methods used for global protein identification - “shotgun”, “bottom-up” and “top-down”-proteomics (Fig. 2). In “shotgun”-proteomics the whole cell lysate or tissue is digested and the internal peptides are then separated by high resolution one or two dimensional nanoHPLC coupled with ESI-MS/MS. In contrast, in “bottom-up”-proteomics the proteins are separated prior to proteolytic digestion and the internal peptides of proteins are used to search genomic databases for protein identification. In “top-down”-proteomics, the biological material will not undergo a proteolytic digestion (Wehr, 2006). Herein, the intact proteins are directly separated by HPLC and then CID is performed in order to obtain fragments of the proteins, which deliver sequence information. The intact masses of proteins together with MS/MS spectra are then used for identifying and characterizing proteins. In the “top-down”-approach special tailored software is needed. Additionally, high resolution separation methods combined with high resolution mass spectrometers are necessary to obtain reliable results in “top-down”-proteomics. The “bottom-up” approach is the most widely used strategy followed by “shotgun”-proteomics. The “top-down”-proteomics is employed much less than the other approaches.

2.3.3.1 Sample pre-treatment prior to mass spectrometry

Following separation by 1-DE or 2-DE and visualization of the bands/spots the proteins are either manually excised or picked by robots. Destaining prior to further steps is recommended. The spots/bands are washed and the proteins are reduced followed by alkylation. The gel pieces are dried using acetonitrile and the proteins are *in-gel* digested using proteases, mostly trypsin (Fig. 9). The resulting peptides are extracted from the gel and then analyzed by using off-line methods such as MALDI-TOF or are further separated by a chromatographic method such as nanoHPLC coupled with ESI-MS/MS. In case of MALDI analysis the peptide solution needs to be desalted using a miniaturized reverse phase chromatography. For shotgun proteomics the cell lysate is digested in solution including a reduction and alkylation step prior to the proteolytic digest.

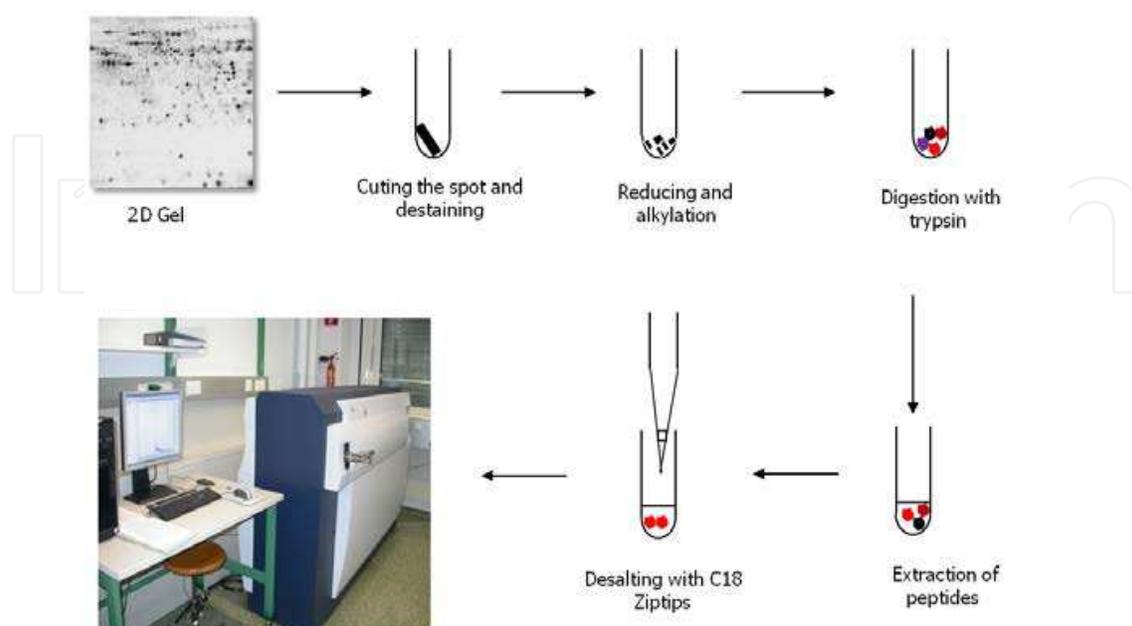


Fig. 9. Workflow and treatment of the gel spots prior to MS analysis.

2.3.3.2 Protein identification by MALDI-TOF/TOF

For protein identification, peptide mass fingerprint (PMF) and tandem mass spectra have to be obtained. PMF is based on the determination of internal peptide masses, which are specific for each protein due to its sequence (Wilkins et al., 2007). It is a rapid and efficient strategy for identifying proteins which nevertheless shows also a number of limitations due to uncertainties. As a consequence MALDI-TOF/TOF instruments have been developed which are able to perform MS/MS. Nowadays it is very easy and fast to analyze post source decay (PSD) ions in order to evaluate the PMF identification results using this type of instruments. It is also possible to obtain sequence information or to perform *de novo* peptide sequencing by collision-induced dissociation (CID).

2.3.3.3 Protein identification by on line LC-ESI-MS/MS

In tandem mass spectrometry parent ions (ionized peptides) will be selected in a first analyzer (MS1) followed by CID with a neutral gas. The fragmented daughter ions will then be separated in a second analyzer (MS2) and detected afterwards (Fig. 11). In the case of peptides the CID will result in specific fragmentation of the polypeptide backbone (Westermeier et al., 2008). The singly charged peptide fragments deliver valuable sequence and structural information. An alternative to PMF is the identification of proteins based on "Peptide Fragment Fingerprinting" (PFF) by nanoHPLC-ESI-MS/MS. In this approach MS/MS spectra are taken into account (Wilkins et al., 2007). Also many post translational modifications like phosphorylation as well as glycosylation of proteins can be studied. In an ion trap the CID fragmentation can be performed in the same trap but hybrid instruments need to have a collision cell.

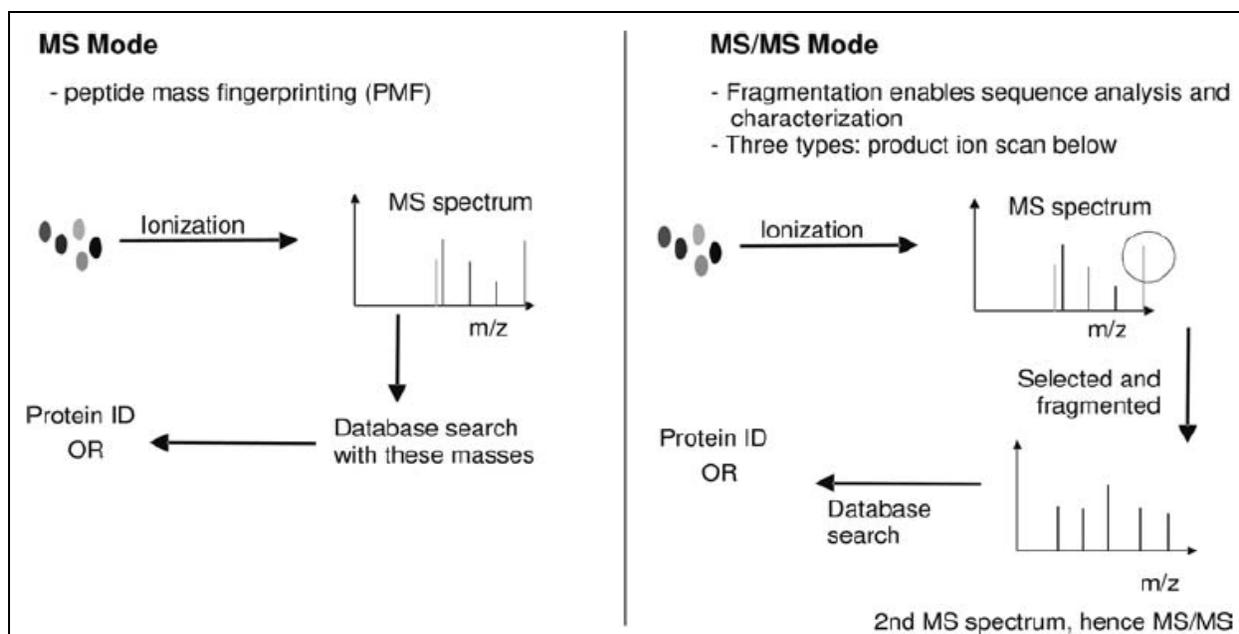


Fig. 10. Schematic illustration of protein identification using MS and MS/MS mode from (Westermeier et al., 2008).

2.3.3.4 Database search

Peptide mass fingerprint or sequences obtained from tandem mass spectra will be matched to virtual protein sequence information in various databases such as Swiss-Prot, which

contain information of annotated protein sequences. Search engines like Mascot or Sequest are used (<http://www.matrixscience.com> , <http://fields.scripps.edu/sequest/>). However, in the case of some fungi there are only few proteins available in protein databases so *de novo* peptide sequencing needs to be performed. With the obtained information protein homology searches such as MS homology, SPIDER, MS BLAST etc. can be carried out (Rizwan et al., 2010).

In the last decade a number of instrumental progresses have led to enormous improvements in biological mass spectrometry. More sensitive instruments with higher resolution and mass accuracy have been introduced. Nevertheless, technical and biological variances should be taken into account, while performing proteomic techniques.

3. Proteomic approaches in aflatoxin research

Contaminations of agricultural commodities with aflatoxins are not only a serious health risk to humans and animals but also result in economic losses. Therefore, strategies were taken into account to reduce the post- and pre-harvest aflatoxin contamination. There are many factors like nutritional and/or environmental signals, which influence aflatoxin biosynthesis. It has been shown that also the production of aflatoxins is highly dependent not only on *Aspergillus* species but also on host plants (Bhatnagar et al., 2008). In addition, drought stress and pre-harvest aflatoxin contamination were studied by analyzing defense and stress-related proteins in plant tissue. In some studies *Aspergillus flavus* infection of corn was associated with the expression of stress-related proteins and antifungal proteins. There are some review papers dealing with the application of proteomics in studying fungal biology (Kim et al., 2008). Additionally, Bhatnagar et al. have reviewed the potential of OMICS technologies including genomics, proteomics, metabolomics for solving aflatoxin contamination problem (Bhatnagar et al., 2008). In a further mass spectrometry-based proteomics study fungal cell wall glycoproteins have been analysed (Yin et al., 2008). Nutritional factors as well as environmental influences play a significant role in effecting

Year	<i>Aspergillus</i> species	No. of separated peptides	No. of identified peptides	Reference
2001	<i>A. fumigatus</i>	19	9	(Bruneau et al., 2001)
2002	<i>A. nidulans</i>	200	5	(Melin et al., 2002)
2004	<i>A. flavus</i>	110	22	(Medina et al., 2004)
2005	<i>A. flavus</i>	>50	51	(Medina et al., 2005)
	<i>A. nidulans</i>	NA	2	(Ström et al., 2005)
2006	<i>A. fumigatus</i>	51	26	(Asif et al., 2006)
	<i>A. oryzae</i>	>110	29	(Oda et al., 2006)
	<i>A. fumigatus</i>	>800	37	(Kniemeyer et al., 2006)
	<i>A. fumigatus</i>	180	54	(Carberry et al., 2006)
2007	<i>A. nidulans</i>	927	30	(Kim et al., 2007 b)
2009	<i>A. fumigatus</i>	-	-	(Kniemeyer et al., 2009)
	<i>A. fumigatus</i>	>700	78	(Zhang et al., 2009)
2010	<i>A. ochraceus</i>	>500	26	(Rizwan et al., 2010)

Table 1. Timetable of proteomics studies in *Aspergillus* species

aflatoxin biosynthesis. One of these factors is drought stress, which is an important factor in preharvest contamination of agricultural commodities with aflatoxins. The conventional breeding, genetic and system biology approaches are valuable tools in improving crop resistance to drought stress resulting on a better aflatoxin management and control strategies. Other approaches have applied proteomics for studying *A. flavus* secreted proteins, effect of environmental signals such as temperature on regulation of proteins in *A. flavus* and studying hepatocarcinogenicity of aflatoxins. In this part we discuss the potential of proteomics to investigate the aflatoxin problematic.

3.1 Proteomics in studying host resistance mechanism to *Aspergillus flavus*

One of the approaches in the field of aflatoxin research with regard to proteomics was to study the aflatoxin resistance proteins in host plants such as corn. The investigation on proteins associated with host resistance has been shown to be a possible strategy for controlling aflatoxin contamination of plants (Brown et al., 2010). An early paper was published by Chen and his co-workers analysing proteins of different corn genotypes, which were found to be resistant or susceptible to *Aspergillus flavus* (Chen et al., 1998). The authors have identified a protein with molecular weight of 14-kDa in relatively high concentrations in kernels of resistant corn genotypes. The protein showed 100% homology of N-terminal sequence to a corn trypsin inhibitor described in a previous work (Swartz et al., 1977). In susceptible hybrids the mentioned 14-kDa protein was not present or only in low concentrations. After the protein extraction and purification by an affinity column using type III trypsin, 1-DE was performed, showing high levels of a 14-kDa protein band in resistant genotypes. The authors suggested that the 14-kDa protein may be useful as a selectable marker for resistance to *A. flavus*.

In a further study the same authors (Chen et al., 2002) performed a proteomics approach to identify potential markers in maize resistant to *A. flavus*. The authors used 2-DE to discover variations in kernel protein expression between genotypes resistant and susceptible. Eleven protein spots, that were upregulated or only present in the resistant maize, were subjected to ESI-MS/MS and conventional Edman degradation for peptide sequencing. From each spot 2 to 9 peptides were sequenced to positively identify the proteins. Based on peptide sequence homology, spots were identified as globulin 1 and 2, which can be categorized as storage proteins. Furthermore, late embryogenesis abundant proteins (LEA) associated with drought were identified. Additionally, heat, water, and osmotic-stress related proteins (aldose reductase, WSI18 and HSP16.9) could be identified.

As *A. flavus* infects crops prior to harvest, the most explored strategy is the investigation of pre-harvest host resistance. One of the methods contributing to the development of host resistance is a kernel screening assay, which shows some advantages compared to traditional breeding methods. Brown et al., 2006 have reviewed efforts and methods dealing with resistance in corn. Using proteome analysis and subtractive approaches several proteins associated with resistance could be identified and characterised. Another review was published by Chen et al. dealing with the identification of aflatoxin resistance-related proteins and genes by using proteomics and RNAi gene silencing (Chen et al., 2009).

The same group of authors has studied the negative correlation between the expression of pathogenesis-related protein (PR10) and the kernel resistance against *A. flavus* infection (Chen et al., 2010). Using an RNAi gene silencing vector in maize embryos the expression of PR10 was reduced in transgenic kernels. Changes in fungal colonization were detected and aflatoxin production was significantly increased. Interestingly, a proteomic approach was

conducted in order to proof the RNAi-silenced kernels. The authors could observe a significant reduction in PR10 production on protein level in RNAi-silenced lines.

In a previously published paper Brown et al., 2010 have reviewed different approaches to identify and characterize proteins associated with aflatoxin resistance in kernel. This review emphasizes that the use of proteomics enabled the identification of a number of aflatoxin resistance associated proteins. Some of them were mapped to chromosomal locations. Additionally, genomic approaches were applied and by QTL (quantitative trait locus) mapping some chromosomal regions, which are associated with low aflatoxin accumulation were found (Brown et al., 2010). The investigations on aflatoxin resistance associated proteins and the regulation of their expression will lead to development of aflatoxins resistant plants. In the future the resistance associated proteins can be used as novel breeding markers.

In a further study Chen et al., 2007 have identified proteins in maize kernel endosperm, which are responsible for resistance to aflatoxin contamination. In resistant lines ten protein spots have been found to be more than two-fold upregulated in comparison to susceptible lines. The following proteins were identified by combination of 2-DE and ESI-MS/MS: globulin-2 protein, late embryogenesis abundant proteins (LEA3 and LEA14), a stress-related peroxiredoxin antioxidant (PER1), heat-shock proteins (HSP17.2), a cold-regulated protein (COR), and an antifungal trypsin-inhibitor protein (TI) (Chen et al., 2007).

Another factor affecting the agricultural commodities is the drought stress. In fact the preharvest contamination of maize, peanuts and other products with aflatoxins has been observed to be higher especially in the drought years, having devastating economical loses. In a recently published review (Guo et al., 2008) have investigated the potential of genetics, genomics and proteomics in understanding the relationship between drought stress and preharvest aflatoxin contamination in agricultural products. In this paper, factors affecting aflatoxin production, host resistance and the tools used to study the responses to drought stress are reviewed. Furthermore, the application of corn proteomics and the relationship to host resistance are discussed. In addition, the function and expression of storage and stress-related proteins, which may enhance the stress tolerance of host plants, were highlighted. Different proteomic approaches revealed that resistant lines have elevated levels of stress-related proteins, antifungal and storage proteins in comparison to susceptible lines (Chen et al., 2002; Guo et al., 2008).

Using proteomic tools a number of proteins could be separated by 2-DE and were further identified using mass spectrometry. Different categories of proteins were found, like resistance associated proteins which were divided into 3 groups: stress-responsive proteins, storage proteins and antifungal proteins indicating that storage and stress-responsive proteins may play an important role in enhancing stress-tolerance of host plant (Chen et al., 2002; Bhatnagar et al., 2008).

Based on research done in this field, proteomic approaches can help scientists to understand mechanisms involved in host resistance as well as stress. The achieved results will lead to new strategies for improving plant resistance against fungal contamination. The discovery of storage and stress related proteins as biomarkers of plant tissues, with regard to aflatoxins, will help breeders to find appropriate strategies to improve plant resistance and stress tolerance of host plants.

3.2 Fungal proteomic approach studying secreted proteins in *Aspergillus flavus*

Filamentous fungi normally secrete a broad spectrum of structural proteins and enzymes into their environment that play amongst others an important role in the nutrition of the mycelia. This fact is used in the biotechnology industry for the production of enzymes needed in food, feed and pharmaceutical industry. The analysis of secreted proteins of *Aspergillus flavus* was performed in order to investigate enzymes and other proteins in this fungus. Additionally, analysis of the fungal extracellular proteome can lead to a better understanding of the pathogenicity of these organisms. In general, proteomics of filamentous fungi is still a relatively new approach. There are only some papers dealing with secretome analysis of filamentous fungi. Most of them have focused on purification and characterisation of single proteins. However there are very little investigations on the global analysis of fungal extracellular proteome.

One of the first papers applying a proteomic approach was published by Medina et al. The authors analyzed rutin-induced secreted proteins of *A. flavus* (Medina et al., 2004). In this study, proteomic analysis to identify the extracellular enzymes of *A. flavus*, grown on the flavonoid rutin as the only source of carbohydrates, were used. 1-DE and 2-DE were used to separate the secreted proteins. The protein spots were analysed by MALDI-TOF MS, where thereby 15 rutin-induced and 7 non-induced proteins could be identified. However, more than 90 protein spots had no positive matches in the database and remained unidentified. There is just very little genome sequence information available for *A. flavus*, making the protein identification difficult.

In a further study Medina et al. have analyzed the secreted proteins from *A. flavus* (Medina et al., 2005), which was grown in rutin-containing, glucose-containing and dextrose-containing media. Culture broths from all three media were collected after 3, 7, 10 and 14 days. After sample preparation, the proteins were separated by 1-DE and 2-DE. Differentially secreted proteins were then further analysed by a nano-HPLC system combined with an ion trap MS. Under three different substrate conditions, 51 unique proteins could be identified. 27% of them were proteins with known function involved in carbohydrate metabolism and 22% were proteins involved in proteolysis and peptidolysis. There were also two identified proteins in the category of redox enzymes and two proteins in the category of electron/proton transport proteins. Due to the lack of available genomic sequences and protein sequences in databases, most of the identifications were performed by using homology BLAST searching. The protein identification will be easier as soon as more genome sequences are available.

Recently a review was published by (Bouws et al., 2008) dealing with different aspects, including the relevance of fungal secretome analysis in biotechnology with regards to secreted enzymes. This indicates that the study of secreted proteins will become an important field of fungal proteomics in the future.

3.3 Quantitative proteomics using stable isotope labelling amino acids in cell culture (SILAC) as a new strategy in studying *Aspergillus flavus* biology

The biosynthesis of mycotoxins in fungi has been shown to be highly dependent on their environment. It is due to the regulation of enzymes involved in biosynthesis pathways of these toxins. In an interesting study, changes in response to environmental stimuli were investigated in fungus *A. flavus* (Georgianna et al., 2008). In this approach temperature-dependent regulation of proteins in *A. flavus* were investigated by using stable isotope labelling of amino acids in the growing culture.

Stable isotope labelling amino acids in cell culture (SILAC) is normally used for relative quantification of protein levels. Mostly the amino acids arginine or lysine are labelled by ^{13}C or ^{15}N isotopes. As the proteolytic enzyme trypsin cleavages proteins on these two amino acids sides, at least some peptides will be labelled N-terminal. In this study authors have performed a modified SILAC procedure using $^{13}\text{C}_6$ -arginine for labelling. The effect of conducive (28°C) and non-conductive (37°C) temperatures for aflatoxin biosynthesis was studied. The cultures in two different approaches were compared: at 28°C for $^{13}\text{C}_6$ -arginine versus to 37°C with $^{12}\text{C}_6$ -arginine and 28°C for $^{13}\text{C}_6$ -arginine versus 28°C for $^{12}\text{C}_6$ -arginine.

Fungal mycelium was harvested and proteins were extracted. After the sample preparation, 1-DE was performed to separate proteins. Each lane was divided into forty bands, which were cut from the gel. Proteins were *in-gel* digested with trypsin and the internal peptides were extracted from the gel. The resulting peptides were separated by nano-HPLC and were analysed using a high resolution mass spectrometer, a linear ion trap combined with a Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FTICR-MS). At 28°C about 18 proteins were found to be upregulated whereas at 37°C 31 proteins were higher expressed. In this study a satisfactorily labelling efficiency of $78 \pm 6.6\%$ was achieved and the fungal organism accumulated the labelled arginine very fast (Georgianna et al., 2008).

Additionally, the authors performed a transcriptomics approach with the same fungal cultures in order to verify the proteomics results and studied the effect of temperature on gene transcription. RNA quantity was determined by RNA microarray and the correlation of gene transcription with protein expression data was studied. The protein expression was observed to have only a moderate correlation between transcript and protein levels for the same gene. At 37°C the changes in enzyme expression involved in aflatoxin biosynthesis were dependent on the repression of the transcription of aflatoxin pathway genes (Georgianna et al., 2008).

In a further approach the same group of authors have performed “top-down”-proteomics combined with SILAC (Collier et al., 2008). Two cultures of *A. flavus*, one grown in a standard medium with $^{13}\text{C}_6$ -arginine, the other in medium with $^{12}\text{C}_6$ -arginine, at 28°C for 24 h, were prepared. Fungal mycelium was then separated by filtration and lysis was performed by grinding with liquid nitrogen. The lysates of both cultures were combined in a 1:1 mixture. The mixture of intact proteins was then analysed using a nano reversed-phase HPLC coupled to a LTQ-FTICR mass spectrometer. In total 1318 intact proteins or fragments were detected corresponding to 659 SILAC pairs of which 22 proteins could be identified. The authors have reported that an incorrect quantification was observed in the case of proteins with greater numbers of arginine, while proteins with fewer arginine had consistent quantification. The labelling efficiency was similar to previous study published by the same authors, being 75-85%.

These initial approaches could clearly demonstrate the potential of quantitative proteomics using SILAC in studying the effects of environmental signals on fungal cells. It could be shown that changes in protein patterns are really measurable. However, additional investigations are needed in order to study the influence of different growth conditions, not only temperature, but also light, agricultural commodity or nutrition as well as antifungal agents on cell biochemistry.

3.4 Proteomics in studying aflatoxin induced hepatocarcinogenesis

Aflatoxins have been shown to be potent carcinogens and liver toxins inducing hepatocellular carcinoma. The consumption of high amounts of aflatoxin over a long period are associated with liver cirrhosis and/or primary liver carcinomas (Seow et al., 2001; Reiter et al., 2009). Especially aflatoxin B1 is a potent carcinogenic agent which undergoes metabolic activation by cytochrom P450 isoenzymes. The products are the carcinogen aflatoxin B1-8,9-exo-peroxide and the DNA adduct N7-guanin- aflatoxin B1 (Li et al., 2008).

A recent paper investigates the carcinogenic effect of aflatoxins on mammalian cells by analysing the different protein pattern of liver biopsies from animals exposed to aflatoxin B1. The liver proteomes before and after induced hepatocarcinogenesis were analysed in order to identify proteins responsible for hepatocellular carcinoma (Li et al., 2008).

In this study hepatocellular carcinoma was induced in adult tree shrews (*Tupaia belangeri chinensis*). Liver tissue of control and treated animals as well as human liver tissues with and without hepatocellular carcinoma were analysed in the same way. The proteomic approach included the separation of proteins by 2-DE followed by MALDI-TOF/TOF mass spectrometric identification of internal peptides. About 1200 spots on every gel could be detected. Differentially expressed proteins were analysed and those proteins (123 spots), with more than two-fold altered expression levels, could be identified. The results revealed that the expression of peroxiredoxin II was upregulated in hepatocellular carcinoma tissue both in tree shrew and in human samples. The authors confirmed the proteomics results by RT-PCR and Western blot. Peroxiredoxins are antioxidant proteins, which protect the cell against oxidative stress (Li et al., 2008).

Such findings contribute to better understanding the mechanism of hepatocarcinogenesis, when investigating the key proteins responsible for tumorigenesis.

4. Conclusion

The application of "OMICS" technologies especially proteomics in mycotoxin research and mycotoxicology is still at the beginning if compared to biomedical approaches. In the recent years there is however an enormous increase of publications in this field. In mycotoxicology, the study of effects of environmental signals on the expression of proteins will become one of the major issues, since mycotoxin production has been shown to be dependent on the growth and cultivation conditions. The complexity of proteomics is quite higher than those of genomics due to the fact that the proteome changes according to environmental signals and other factors influencing the cell. Additionally, proteins are processed within the cell by post translational modification, ubiquitination and proteolytic degradation. Proteomic approaches require very expensive equipments and highly trained scientists. Many developments, especially in the field of mass spectrometry, will help to overcome problems regarding sensitivity and mass accuracy as well as mass resolution. It will also contribute to better and reliable protein identifications as well as quantification in proteomics. A further challenge is the systematic and efficient analysis of vast data, which needs suitable bioinformatic tools.

In future the combination of major OMICS technologies like genomics, transcriptomics, proteomics and metabolomics will significantly accelerate the understanding of fungal cell life, its secondary metabolite machinery and cellular responses to its environment. As a consequence the achieved knowledge will help to prevent and/or reduce mycotoxin contamination in agricultural commodities.

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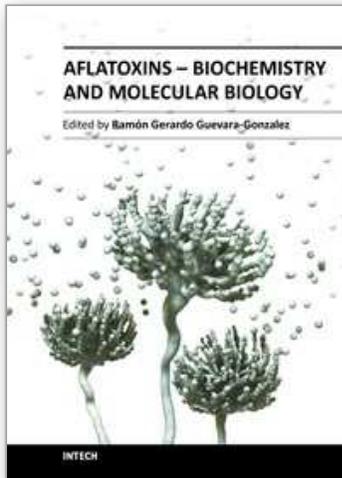
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Aflatoxins – Biochemistry and Molecular Biology is a book that has been thought to present the most significant advances in these disciplines focused on the knowledge of such toxins. All authors, who supported the excellent work showed in every chapter of this book, are placed at the frontier of knowledge on this subject, thus, this book will be obligated reference to issue upon its publication. Finally, this book has been published in an attempt to present a written forum for researchers and teachers interested in the subject, having a current picture in this field of research about these interesting and intriguing toxins.

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