

REVIEW

Proteomics of eukaryotic microorganisms: The medically and biotechnologically important fungal genus *Aspergillus*

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Fungal species of the genus *Aspergillus* play significant roles as model organisms in basic research, as “cell factories” for the production of organic acids, pharmaceuticals or industrially important enzymes and as pathogens causing superficial and invasive infections in animals and humans. The release of the genome sequences of several *Aspergillus* sp. has paved the way for global analyses of protein expression in *Aspergilli* including the characterisation of proteins, which have not designated any function. With the application of proteomic methods, particularly 2-D gel and LC-MS/MS-based methods, first insights into the composition of the proteome of *Aspergilli* under different growth and stress conditions could be gained. Putative targets of global regulators led to the improvement of industrially relevant *Aspergillus* strains and so far not described *Aspergillus* antigens have already been discovered. Here, I review the recent proteome data generated for the species *Aspergillus nidulans*, *Aspergillus fumigatus*, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus flavus* and *Aspergillus oryzae*.

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1 Introduction

Fungi make a significant contribution to our life. We consume fungi directly or they are used as a component in food-making processes. Besides, they play an important role in the field of white biotechnology. In their natural habitat, they represent important mutualistic symbionts, parasites or primary decomposers of organic material [1]. On a more negative note, plant-pathogenic fungi are responsible for important crop losses worldwide [2]. A limited number of fungi are also capable of causing superficial or severe, often lethal, invasive infections in humans [3]. However, most

fungi are living in obscurity and of the estimated total number of 1.5 million fungal species only approximately 100 000 species have been described to date [4].

This review will merely focus on the best studied and most important species of the filamentous fungal genus *Aspergillus* [5], which are described in the following overview. The species *Aspergillus nidulans* is one of the most well-studied model organism for filamentous fungi and has already been intensively investigated since the 1950s to explore fungal genetics and cell biology [6, 7]. Research on *Aspergillus fumigatus* has gained increasing interest because it is the main cause for airborne opportunistic fungal infections in humans, including particular the often fatal infection invasive aspergillosis (IA) (reviewed in [8–11]). The second most frequent pathogenic species is *Aspergillus flavus* and to a lesser extent *Aspergillus terreus*, both of which are able to cause superficial and invasive mycoses [12–14]. Additionally, *A. flavus* produces aflatoxins, very potent toxic and hepatocarcinogenic natural compounds. Hence, this *Aspergillus* sp. poses also threats to foods and feeds by contaminating stored grain and other economic plants with

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Abbreviations: ABPA, allergic bronchopulmonary aspergillosis; FB₂, fumonisin B₂; GPI, glycosylphosphatidyl inositol; IA, invasive aspergillosis; PKA, protein kinase A

aflatoxins [15]. *A. terreus* is also famous for its production of the bioactive, cholesterol-lowering metabolite lovastatin [16]. The species *Aspergillus oryzae* is closely related to *A. flavus*, but does not secrete toxic mycotoxins and has rather been used for more than a millennium in the traditional Japanese fermentation industry to produce rice wine, soy sauce and soybean paste [17]. The industrially most important *Aspergillus* sp. worldwide is for certain *Aspergillus niger*. It is used as “cell factory” for manufacturing million tons of citric acid per annum and a wide range of commercial enzymes [18].

The recent releases of the genome sequence of the abovementioned *Aspergillus* sp. (Table 1) have paved the way for global studies and ushered in the post-genomic era in *Aspergillus* research [19–25]. In comparison to transcriptome research (e.g. [26, 27]; reviewed in [28]), the number of global protein expression studies on *Aspergillus* is relatively low. The majority of the *Aspergillus* proteome research is still represented by quantitative 2-DE studies, whereas LC-MS/MS-based techniques have only rarely been used (reviewed in [28–32]). Roughly estimated, < 10% of the predicted whole proteome of *Aspergillus* sp. has been identified and quantified in published proteomic studies until now. Thus, proteome research on filamentous fungi like *Aspergillus* sp. clearly lag behind the deep proteome analyses of *Saccharomyces cerevisiae* and other microbial model organisms with identification of around two-third of the predicted open reading frames [33, 34]. However, *Aspergillus* proteomics is currently catching up and it is safe to say that modern proteomic approaches definitely hold great potential to progress the level of knowledge in the field of *Aspergillus* proteome profiling. Since the introduction of the combination of multidimensional chromatography of peptides (generated after proteolytic digestion of proteins) and subsequent tandem mass spectrometry, the detection of classes of proteins which are underrepresented on 2-D gels has become possible, such as very hydrophobic proteins, membrane proteins and proteins with extreme pI [35].

The aim of this review is to survey the current status of proteome profiling in *Aspergilli* and to emphasise the great potential modern proteome methods offer to foster

Aspergillus basic and applied research. I begin by providing an overview about the classes of proteins, which have already been detected and highlight new findings in the field of fungal physiology, the environmental stress response, *Aspergillus* biotechnology and pathogenicity.

2 *Aspergillus* proteome and subproteome mapping

In the pre-genomic *Aspergillus* era, *A. nidulans* was almost exclusively object for studies using 2-DE as a method to explore basic concepts in fungal biology, for example, to study the role of tubulins in fungal development [36]. After having the *Aspergillus* genome sequence available, protocols for *Aspergillus* proteomics were developed to investigate the intracellular protein composition of *Aspergillus* sp., but included also the survey of subcellular proteomes, in particular of secreted, mitochondrial and membrane proteins.

2.1 Sample preparation and employed techniques

Proper sample preparation is a critical point in proteome analyses. However, it is often the most overlooked step in achieving high-quality results. First of all, the fungal cells have to be disrupted, which is hampered by the fact that the fungal cell wall is exceptionally robust. Later, proteins need to be sufficiently solubilised in a buffer that is compatible with 2-DE or MS analysis. Sample preparation protocols for fungi were tested and introduced by several groups. In most cases, mechanical lysis of mycelium or spores was preferred over chemical or enzymatic disruption methods [37]. For 2-DE, fungal protein extracts are often subsequently purified by trichloroacetic acid or methanol chloroform precipitation or, alternatively, by phenol extraction followed by protein pelletation with ammonium acetate in methanol. Such treatments allow the removal of substances, e.g. pigments, lipids and polysaccharides, which interfere with subsequent electrophoretic protein separations [38–41]. Most labora-

Table 1. Overview about the *Aspergillus* sp. which have been studied by proteomic methods

	Genome size ^{a)} (kb)	Gene models	Intracellular proteome	Secretome	Cell wall proteome	Membrane proteome	Subproteome	Stress response
<i>A. fumigatus</i> ^{b)}	29.4 29.2	9887 10 099	✓	✓	✓	✓	Mitochondria, glutathione binding	ROS, heat, hypoxia
<i>A. niger</i> ^{b)}	33.9 34.9	14 165 11 200	✓	✓	–	–	Microsomes	–
<i>A. flavus</i>	36.3	13 071	✓	✓	–	–	–	Heat
<i>A. oryzae</i>	37	12 074	–	✓	–	–	–	–
<i>A. nidulans</i>	30.1	10 701	✓	–	✓	–	–	ROS, osmo, hypoxia, -Fe
<i>A. terreus</i>	29.3	10 406	–	✓	–	–	–	–

a) Data were obtained from [28].

b) Two strains of *A. fumigatus* and *A. niger* have been sequenced.

tories employed immobilised pH gradients strips for the isoelectric focusing of proteins in the first dimension followed by SDS-PAGE for the separation of proteins according to their molecular mass in the second dimension [42]. For the subsequent detection of proteins, either classical post-staining methods were applied (Coomassie blue, silver staining, fluorescence stains) or proteins were labelled prior to electrophoresis with spectrally resolvable fluorescent dyes. This multiplexing approach designated as differential gel electrophoresis (DIGE) reduces gel-to-gel variations of protein spot patterns and provides statistically more reliable data [43]. A limited number of laboratories applied LC-MS/MS-based methods [44] for the separation of *Aspergillus* protein extracts: here, proteins were separated by SDS-PAGE, and bands were excised from each gel lane and subsequently tryptically digested. Then, peptide mixtures of each in-gel-digested sample were separated by LC and subsequently eluted into a mass spectrometer. In some experiments, metabolic labelling by using isotopically enriched media was applied for the quantitation of proteins. In this case, growth medium is supplemented with either an isotopically light substrate or the heavy counterpart, so that the different isotopes, e.g. ^{14}N and ^{15}N , are incorporated into all proteins during growth. Owing to the mass shift, the abundance of proteins or peptides, respectively, can be compared and quantified between two samples [45–47]. Stable isotopes can also be introduced to a later time point after preparation of the protein extracts by chemical binding of an isotopic tag to proteins or peptides. For example, Cagas et al. [48] applied a labelling technique known as iTRAQ (isobaric tag for relative and absolute quantification) to investigate the response of *A. fumigatus* to the antifungal drug caspofungin [49].

2.2 Intracellular proteins

Proteome maps give an impression of the kind and quantity of proteins detectable by 2-D gel or LC-MS/MS approaches. Furthermore, two recent publications highlighted the potential of proteomic data to improve gene annotation and to discover alternative splice variants of transcripts and isoforms of proteins [45, 47]. For *A. fumigatus*, two studies characterised the mycelial intracellular proteome of *A. fumigatus* and identified 54 and 334 proteins, respectively [50, 51]. In a quite similar manner, Lu et al. [52] identified a little more than 100 cytosolic proteins in *A. niger*. In all studies, proteins primarily involved in translation, energy metabolism, transport processes and the stress response were most abundant. This also holds true for the spore proteome. The asexually produced spores of *Aspergilli*, designated as conidia, represent the dispersal units and, for pathogenic fungi, the infectious agent, which can easily be inhaled by humans. Teutschbein et al. [41] characterised the proteome of dormant conidia of *A. fumigatus*. They contained in particular high amounts of proteins, which are required for stress

tolerance and rapid reactivation of metabolic processes. The authors speculated that such a protein composition reflects the enormous stress resistance of conidia and their ability to start germinating within a couple of hours. In another study, a comparison of the proteome of germinating conidia with hyphae of *A. fumigatus* revealed that a CipC-like protein was one of the major hyphal-specific proteins. However, the biological function of this cytosolic protein has not been elucidated yet, but a putative role during invasive growth in the host is discussed [53]. Similar results were obtained for *A. nidulans* [54]. During the first hour of conidial germination levels of proteins involved in metabolism, protein synthesis and transcription highly increased, confirming the importance of metabolic activation and new protein synthesis for the germination process.

Altogether, the generation of 2-D gel reference maps for *Aspergillus* sp. illustrated that especially high-abundant proteins involved in metabolic processes and the general stress response are easily accessible to gel-based proteomic approaches, but demonstrated also the limitation of this technique to separate hydrophobic, membrane and low-abundant proteins.

2.3 Secreted proteins

Saprophytic fungi like *Aspergillus* sp. produce a diverse set of extracellular enzymes, the secretome, which enable them to adapt their metabolism to varying carbon and nitrogen sources. These extracellular proteins have significant relevance for the biotechnology industry due to their specific enzymatic activities, but they certainly play also a role during infection or as allergens in pathogenic *Aspergilli*. Thus, it is no surprise that many proteomic studies were conducted to characterise the extracellular proteome of *Aspergilli*.

The first global surveys on secreted proteins of *Aspergillus* sp. were initiated prior to the release of annotated genome data of *Aspergillus* sp. Schwenbacher et al. [55] analysed the major secreted proteins of *A. fumigatus* and compared the protein pattern on SDS-PAGE gels with those obtained from other *Aspergillus* sp. The major secreted proteins of *A. fumigatus* were identified as the ribonuclease mitogillin, interestingly an inhibitor of eukaryotic protein synthesis, and two cell wall polymer-degrading enzymes, the chitosanase chiB and a β -1,3 endoglucanase. Remarkably, anti-chitosanase and anti-mitogillin antibodies were detected in sera of patients suffering from IA or aspergilloma, which opened up the perspective to use antibodies directed against secreted proteins as diagnostic marker. Subsequent studies focused on the protease profile of *A. fumigatus*. Neustadt et al. [56] employed free-flow electrophoresis for the separation of *A. fumigatus* proteases and found in fractions with an acidic pH the highest proteolytic activity. A further study emphasised the influence of the pH on the sets of proteases secreted by *A. fumigatus*: neutral pH favoured the secretion of neutral and alkaline endoproteases, in particular the serine protease Alp1

and the dipeptidyl peptidase Dpp5, whereas acidic pH promoted the secretion of a tripeptidyl-peptidase and an aspartic endopeptidase [57]. The prevalence of Alp1 as the main protease even in the absence of a protein substrate was confirmed by Wartenberg et al. (submitted for publication). An interesting point in this line is the degrading activity of Alp1 on human complement regulators and, as a result, its putative role in immune evasion [58].

For *A. flavus*, two pioneering studies of Medina et al. [59, 60] reported the extracellular enzymes secreted by this food-borne pathogen when grown on the plant-produced, anti-microbial flavonoid rutin. Ten proteins were found to be uniquely synthesised during growth on rutin. Particularly noteworthy here is the quercetin 2,3-dioxygenase, which is presumably involved in the degradation of rutin. More recent secretome studies employed either 2-DE or LC-MS/MS-based approaches to identify and profile secreted proteins in *A. terreus* [61], *A. oryzae* [62, 63] and *A. niger* [64–67] during growth on different kinds of substrates. Between 16 and 292 different proteins with diverse functions were identified. These initial secretome studies have opened a door for comparative analyses of fungal secretomes and the following general statements can tentatively be made: (i) especially the growth substrate has a great impact on the type of proteins secreted by *Aspergillus* sp.; (ii) the basal secretome reflects the saprophytic lifestyle of this group of fungi. Under almost each growth condition tested, *Aspergilli* secrete glycosylases, which are required to utilise plant-derived polymers. (iii) Besides the growth substrate (carbon source), the nitrogen source and the pH greatly influence the composition of the secretome; (iv) a certain proportion of the proteins found to be secreted do not have a secretory signal peptide for protein export, which supports the existence of novel secretory mechanisms in filamentous fungi independent of the classical ER-Golgi secretory pathway.

To elucidate the mechanisms underlying protein secretion in filamentous fungi, shotgun proteomics of secretory organelles in *A. niger* was deployed by Ferreira de Oliveira et al. [68]. After the substrate-specific induction of cellulase and hemicellulase enzymes, the most prominent change was the specific recruitment of 20S core particle of the proteasome to the secretory organelles. This result pointed to a regulatory role of the proteasome upon the induction of extracellular enzymes.

Altogether, the characterisation of the secretomes of several *Aspergillus* sp. has made it increasingly obvious that the mechanisms of protein secretion in filamentous fungi are complex, but the knowledge about these processes is rather limited and additional research is highly needed.

2.4 Cell wall and membrane proteins

The fungal cell wall is vital for the maintenance of the cell integrity and for the interaction with the environment (reviewed in [69]). Thereby, cell wall proteins play specific

roles as adhesins, enzymes, allergens or immunomodulators. However, despite their importance, only few studies have targeted the *Aspergillus* cell wall proteome yet. Bruneau et al. [70] identified nine proteins from mycelial cells of *A. fumigatus*, which were linked to the cell membrane via a glycosylphosphatidyl inositol (GPI) anchor. These proteins had been released from isolated membranes by the activity of an endogenous GPI phospholipase. Four of them were identified as enzymes involved in cell wall biogenesis: two glucanosyltransferases (Gel1p and Gel4p) and two glucanases (Crh1p and Crh2p). In another LC-MS/MS-based study of *A. nidulans* cell surface proteins, de Groot et al. [71] found ten predicted GPI-anchored proteins in cell wall fractions of *A. nidulans*. Six of these proteins were specified as cell wall carbohydrate-active enzymes including three orthologous proteins similar to the *A. fumigatus* proteins Gel1, Gel4 and Crh2, which had been described before by Bruneau et al. [70].

Asif et al. [72] characterised the conidial surface proteome and found 26 proteins, which were released upon treatment with the cell wall-degrading enzyme β -1,3-glucanase. Among others, the conidial surface layer protein RodA, the aspartic proteinase PEP2, a lipase and, surprisingly, many proteins without a signal for secretion were identified. The authors speculated that some of the conidial proteins may be important for the interaction with the host after inhalation of spores and could be decisive for the pathogenesis of *A. fumigatus*-related allergic diseases. Indeed, the RodA protein was shown to prevent the recognition of *A. fumigatus* conidia by the immune system [73].

Besides cell wall proteins, also the analysis of membrane proteins forms a major challenge in the field of proteomics. Ouyang et al. [74] separated a membrane preparation from *A. fumigatus*. In total, 530 proteins were unambiguously identified including 17 integral membrane proteins involved in *N*-, *O*-glycosylation or GPI-anchor biosynthesis. On basis of this information, metabolic pathways for *N*- and *O*-glycosylation biosynthesis were predicted. In addition, many sphingolipid and cell wall biosynthesis enzymes were detected, which illustrates the fact that cell wall polysaccharides are synthesised at the membrane level and that the proteins involved in this process are obviously quite abundant.

Despite such progress, the proteomic analysis of cell wall and membrane proteins remains a tough task: cell wall proteins are difficult to separate from cell wall polysaccharides and are often highly glycosylated, which hampers MS analysis. Membrane proteins are usually low in abundance and due to their hydrophobicity, hard to dissolve and difficult to digest by standard methods (hydrophobic parts lack tryptic cleavage sites).

2.5 Subproteomics

To increase the resolution of proteins contained on 2-D gels, sample prefractionation, e.g. the production of subcellular

fractions, is a powerful tool. Apart from producing ATP, mitochondria contribute to many physiological functions in the cell, such as apoptosis, signalling and cellular metabolism. On this account, *A. fumigatus* mitochondria were isolated and a mitochondrial proteome map was established by Vödisch et al. [51]. This subproteomic approach led to identification of 55 additional proteins, which had not been found in a mycelial proteome map before, e.g. many respiratory proteins and enzymes involved in catabolic metabolism and amino acid biosynthesis.

Carberry et al. [50] deployed a sub-proteomic strategy involving glutathione affinity chromatography, to enrich glutathione-binding proteins. After 2-DE separation, the elongation factor eEF1B γ proteins, ElfA and ElfB, were detected and found to exhibit glutathione transferase activity. The authors speculated about the possible role of ElfA and ElfB in protecting against or controlling translation in response to oxidative stress. The above-described results demonstrate the advantage of subcellular fractionation or affinity chromatography techniques. They can give access to lower abundant proteins by depleting other, highly abundant proteins.

3 *Aspergillus* physiology

3.1 The environmental stress response

Many of the niches that *Aspergillus* sp. occupy in the environment do not provide constant, but rather fluctuating environmental conditions. As a result, most filamentous fungi encounter different stresses such as iron depletion, osmotic shock, heat stress and many others. The defence systems mounted by *Aspergillus* sp. against these environmental stresses have most intensively been investigated in *A. fumigatus* and *A. nidulans* by proteomic methods.

The oxidative stress response was analysed in both *A. fumigatus* and *A. nidulans* by 2-DE. In the presence of hydrogen peroxide, proteins with antioxidant functions such as thioredoxin peroxidases, cytochrome *c* peroxidase, Cu/Zn superoxide dismutase and catalase Cat1 were induced in *A. fumigatus*. Similarly, heat shock proteins, enzymes of the pentose phosphate shunt and a spermidine synthase increased in abundance within 45 min [75]. By contrast, the addition of the reactive oxygen-generating compound menadione for 6 h provoked a completely different induction of antioxidative enzymes in *A. nidulans*: the peroxide-detoxifying peroxiredoxins and cytochrome *c* peroxidase were repressed and were replaced by a thioredoxin reductase. Furthermore, enzymes indicative of evolving nitrosative stress and enzymes of the glutathione *S*-transferase family were highly abundant [76]. These results suggest differences in short- and long-time adaptations to oxidative stress in *Aspergilli* or, alternatively, differences in the oxidative stress response between different species.

In various aspects, the heat shock response resembles the adaptation to oxidative stress. Several heat shock proteins

and anti-oxidative enzymes showed an upregulation under both conditions. Albrecht et al. [77] used an optimised data analysis workflow for DIGE to elucidate the mechanisms of heat resistance in the thermotolerant species *A. fumigatus*. During the heat shock response (from 30 to 48°C), the heat shock proteins HSP30/HSP42 and HSP90 showed the highest increase in abundance. In addition, enzymes of the oxidative stress response were induced, presumably because of a higher respiration rate at higher temperatures, which leads to an elevated production of ROS [77].

Since the biosynthesis of the mycotoxin aflatoxin is temperature-dependent, temperature adaptation was also studied in *A. flavus*. Georgianna et al. [78] compared the proteome of *A. flavus* at conductive (28°C) and nonconductive (37°C) temperature for aflatoxin production. As expected, most aflatoxin biosynthesis proteins showed a higher abundance at 28°C; on the other hand, an enzyme of the mevalonate-ergosterol pathway and the protein HSP30, among others, were upregulated at the nonconductive temperature of 37°C. Stress-related heat shock proteins are also induced during osmoadaptation in *A. nidulans*, but especially enzymes involved in the biosynthesis of the osmolyte glycerol increased upon osmotic shock, whereas the level of TCA (tricarboxylic acid) cycle enzymes decreased [79].

Another stress that *A. fumigatus* and other pathogenic fungi may encounter in vivo during infection is a low level of oxygen (hypoxia). To ensure highly reproducible hypoxic growth conditions, an oxygen-controlled chemostat cultivation was established for *A. fumigatus* by Vödisch et al. [80]. 2-DE analysis of mycelial and mitochondrial proteins as well as 2-D Blue Native/SDS-gel separation of mitochondrial membrane proteins led to the identification of 117 proteins with an altered abundance under hypoxic in comparison to normoxic conditions. The proteome data gave no hint to an induction of a fermentative pathway in *A. fumigatus* upon hypoxia. Quite the contrary, many respiratory proteins were upregulated. Furthermore, hypoxia led to the activation of genes and their corresponding protein products, which are involved in the biosynthesis of the secondary metabolite pseurotin A. It was speculated that the stimulation of pseurotin A biosynthesis by oxygen depletion may also affect *A. fumigatus*' survival in the human host. In comparison, *A. nidulans* was shown to reduce nitrate to ammonium under hypoxic conditions when grown on ethanol as a carbon source, which was converted into acetate for ATP generation [81]. The proteomic survey of this "hypoxic metabolism" in *A. nidulans* revealed an induction of the alcohol and aldehyde dehydrogenase for ethanol oxidation under hypoxic conditions, but at the same time, the nucleotide and thiamine metabolism was upregulated. This indicated at a higher level of DNA base damages during hypoxic growth conditions.

The regulatory mechanisms of iron homeostasis were intensively examined in *A. nidulans*. One of the central components of iron regulation in *Aspergilli* is the transcriptional regulator HapX [82]. HapX represses iron-

dependent pathways under iron depleting (-Fe) conditions by interaction with the heterotrimeric CCAAT-binding core complex (CBC) that is involved in redox regulation [83]. The proteome analysis performed on mycelia of an *A. nidulans* wild type and *hapX*-deletion strain grown under iron limitation revealed 30 differently regulated proteins in the $\Delta hapX$ mutant in comparison to the wild-type strain. Among these putatively HapX-regulated proteins, heme-containing enzymes as well as iron-sulphur cluster containing proteins such as the 5-aminolevulinic acid synthase were over-represented. The latter enzyme catalyses the first step in heme biosynthesis. Its induction under -Fe conditions underlined the repressive effect of HapX on iron consuming pathways and the enormous deregulation in iron homeostasis in the $\Delta hapX$ strain.

Another adaptive response to altered environmental conditions in microorganisms is the formation of a biofilm. Also, *A. fumigatus* has the ability to develop a complex growth form of dense intertwined mycelium enclosed in an extracellular matrix, which resembles microbial biofilms. A combined transcriptomic and proteomic study about the *A. fumigatus* biofilm lifestyle revealed an enhanced production of the mycotoxin gliotoxin during “multicellular” growth. The authors speculated that this finding could also play a role in chronic *A. fumigatus* infections, e.g. in cystic fibrosis patients, where the appearance of biofilm-like growth forms of *A. fumigatus* is suspected and an increased synthesis of the immunosuppressive gliotoxin may confer protection from the host innate immune system [84]. The self-protection of *A. fumigatus* against the deleterious effect of gliotoxin is mediated by the disulphide oxidase GliT, an enzyme also involved in gliotoxin biosynthesis [85]. This connection was revealed by the observed increased levels of GliT on 2-D-gels from protein extract of *A. fumigatus* cultures that had been supplemented with exogenous gliotoxin [86].

Also pigments fall into the class of secondary metabolites produced by fungi. 2-DE helped to elucidate the origin of a brown pigment formed during growth of *A. fumigatus* on tyrosine. The induced expression of enzymes of the tyrosine degradation pathway suggested that the accumulation of homogentisate, an intermediate of the tyrosine degradation pathway, led to the formation of pyomelanin. Further detailed genetic studies and chemical analyses enabled to confirm this assumption [87]. It is interesting to note that another pigment produced by *A. fumigatus*, the DHN-melanin, has been shown to be involved in its virulence [88, 89]. The biological significance of pyomelanin formation during the development of an *Aspergillus* infection has still to be elucidated.

3.2 Mutant characterisation

Proteomic profiling of *Aspergillus* mutant strains can help to improve our understanding of how mutant phenotypes emerge and, in addition, can aid to identify new molecular components of regulatory circuits [90]. Several groups analysed

the global protein profiles of mutants of *A. fumigatus* and *A. nidulans*. In many cases, mutant-specific proteomic changes were detected and subjected to further detailed analyses.

In *A. nidulans*, the heterotrimeric CCAAT-binding complex is a global regulator of the redox status of the cell. It consists of the three subunits HapB, HapC and HapE and binds to CCAAT sequences in the promotor regions of a large number of *A. nidulans* genes. Deletion of the *hapC* gene led to an impaired oxidative stress response. This observation was confirmed by comparison of the proteome profiles of the $\Delta hapC$ and *A. nidulans* wild-type strain. Several proteins with altered levels in $\Delta hapC$ extracts had antioxidative functions. The prevailing increased oxidative stress was also illustrated by the fact that the peroxiredoxin PrxA was found to be hyperoxidised in $\Delta hapC$ extracts. MALDI-MS analysis revealed an oxidation of the thiol-group of a specific cysteine to its sulphinic and sulphonic form, which is accompanied by an incorporation of a negative charge and a pI shift of the peroxiredoxin [83].

Redox regulation was also impaired in *A. nidulans* mutants without a functional COP9 signalosome complex. This complex is a crucial regulator of ubiquitin ligases and promotes the sexual development in *A. nidulans*. Deletion of the metalloprotease-containing Csn5 subunit affected the levels of cytoplasmatic proteins sharing a connection to oxidative stress [39].

Another central signalling enzyme was studied in *A. fumigatus* by Grosse et al. [91]: the protein kinase A (PKA). It is a central component of the cAMP signalling cascade and consists of two regulatory (PKAR) and two catalytic (PKAC) subunits. PKA is involved in many cellular processes, such as growth, development, reproduction and infection. To identify proteins of *A. fumigatus* that are regulated by PKA, a transgenic mutant with an ectopic integration of the *pkaC1* gene under control of an inducible promotor was analysed by 2-DE. Elevated PKA activity resulted in activation of stress-associated proteins like chaperones, enzymes involved in pyruvate metabolism and protein biosynthesis, whereas amino acid metabolism and enzymes involved in catabolism of alternative carbon sources other than glucose were downregulated. These results demonstrated the high diversity of cellular processes in *A. fumigatus*, which are under the control of PKA [91].

Mouyna et al. [92] and Zhang et al. [93] investigated *A. fumigatus* mutants impaired in O-mannosylation and N-glycan-processing of proteins, respectively. Disruption of the O-mannosyltransferase PMT4 resulted in abnormal mycelial growth, reduced conidiation and drastic changes of the proteome. Proteins involved in the general stress response, protein folding and stabilisation were most significantly affected by the *pmt4* deletion showing that the lack of PMT4 causes permanent cellular stress. Zhang et al. [93] deleted the α -glucosidase AfCwh41, which plays an important role in N-glycan processing of proteins and thus in the quality control of glycoproteins in the ER. 2-DE analysis revealed an altered level of proteins involved in

protein assembly, ubiquitin-mediated degradation and actin organisation. The authors concluded that blocking of the N-glycan trimming induced accumulation of misfolded proteins in the ER and as a result, an induced expression of chaperones and proteins of the ubiquitin-proteasome pathway. Both studies showed the biological significance of protein glycosylation in fungal moulds.

4 Proteomics for biotechnology

Proteomics also play a significant role in the field of biotechnology and can directly contribute to the optimisation of fermentation processes and to the screening for biotechnologically relevant enzymes.

During industrial fermentations, filamentous fungi like *A. niger* also produce unwanted side products, such as secondary metabolites. The cytotoxic mycotoxin fumonisin B₂ (FB₂) was recently found to be produced by *A. niger*. To evaluate the potential risk of mycotoxin production by *A. niger* during biotechnological fermentations or infestation of foods and feeds with this mould, Sørensen et al. [94] explored the influence of the substrate on FB₂ production. Lactate when added to a medium containing starch synergistically increased the production of FB₂. A 2-DE-based proteome study was conducted to elucidate the connection between the level of secondary metabolite production and the medium composition. Many of the proteins, which showed a different expression on starch/acetate medium, were enzymes that affect the intracellular level of acetyl-CoA and NADPH. The authors concluded that the production of fumonisin is regulated by the intracellular level of acetyl-CoA.

Proteomic methods can also be applied to improve protein production in *A. niger*. An integrated genomics approach was developed to determine the response of *A. niger* to protein production in batch fermentations. Samples were collected for both transcriptome and proteome analysis. Upregulated proteins in enzyme-over-producing strains included proteins involved in carbon and nitrogen metabolism, the oxidative stress response, protein folding and ER-associated degradation (ERAD). Based on these results, several genes were selected as leads for strain improvement. In a first trial, an *A. niger* mutant was constructed with a slightly improved protein production yield that lacked an ER-associated degradation factor and that overexpressed an oligosaccharyl transferase [95].

Proteomics was also employed to discover novel enzymes with either a better activity or stability or specificity. Especially *Aspergillus* sp. are very suitable objects of study for this approach. Kim et al. [96] developed a protocol based on 2-DE and subsequent in situ activity staining to screen for new β -glucosidases in the supernatant of *A. fumigatus* cultures. One of the two newly detected β -glucosidases was heterologously expressed and further characterised: it showed far superior heat stability to previously characterised β -glucosidases and has thus great potential to be used for the

hydrosylation of cellulose to fermentable sugars. Another application for *Aspergillus* enzymes was shown for a proline-specific endoprotease from *A. niger*. It was applied as an alternative to trypsin for the generation of predictable digest peptides for further MS analysis [97].

5 Proteomics and fungal disease

Proteomics also deliver the tools to study the molecular mechanisms of *Aspergillus* pathogenicity. However, at the present stage, studies of the fungal response in vivo remain challenging. Until now, proteomic methods have particularly been used to screen for new fungal antigens and to characterise the response of *Aspergilli* to antifungal agents. In the following sections, the current status will be summarised.

5.1 Immunoproteomics

The identification and characterisation of antigenic proteins is a requirement for the development of antifungal vaccines, which have retained increased interest as an alternative therapeutic treatment of fungal infections. In addition, fungal antigens could also be used as a diagnostic tool. For investigations of the immunoreactivity of fungal proteins with human or murine immunoglobulines (Igs), the combination of 2-DE and immunoblotting still represents the gold standard (Fig. 1). The aim of many research projects has been to screen for immunoreactive anti-*Aspergillus* IgE antibodies, since *A. fumigatus* and other *Aspergilli* are the causative agent of allergic diseases such as allergic bronchopulmonary aspergillosis (ABPA) and allergic asthma. However, due to the increased attention for the development of an immunotherapy for IA also *Aspergillus*-specific IgG antibodies have been in the focus of research. The first attempts to study the serological response to *Aspergillus* antigens were initially carried out in the pre-genomics era and identification of proteins was quite laborious at that time (reviewed in [31]). In more recent studies, more than 50 different *Aspergillus* antigens have been identified. Gautam et al. [98] characterised the secreted antigens from a static-grown *A. fumigatus* culture. The authors used sera of *A. fumigatus*-sensitised asthmatics and identified 11 novel antigens: the most prominent were an extracellular arabinase and a noncharacterised chitosanase. In the study of Glaser et al. [99], the protein PhiA, which is essential for phialide development, was detected as the major antigen recognised by IgE antibodies from sera of ABPA patients. Also, Singh et al. [100] used pooled ABPA patients' sera to identify novel *A. fumigatus* antigens. They found three IgG- and 63 IgE-reactive proteins, which had been extracted from germinating conidia of *A. fumigatus*. In a similar approach, the same authors detected 35 different secreted proteins with IgG/IgE binding capability, of which 25 proteins had been described as *Aspergillus* antigens before

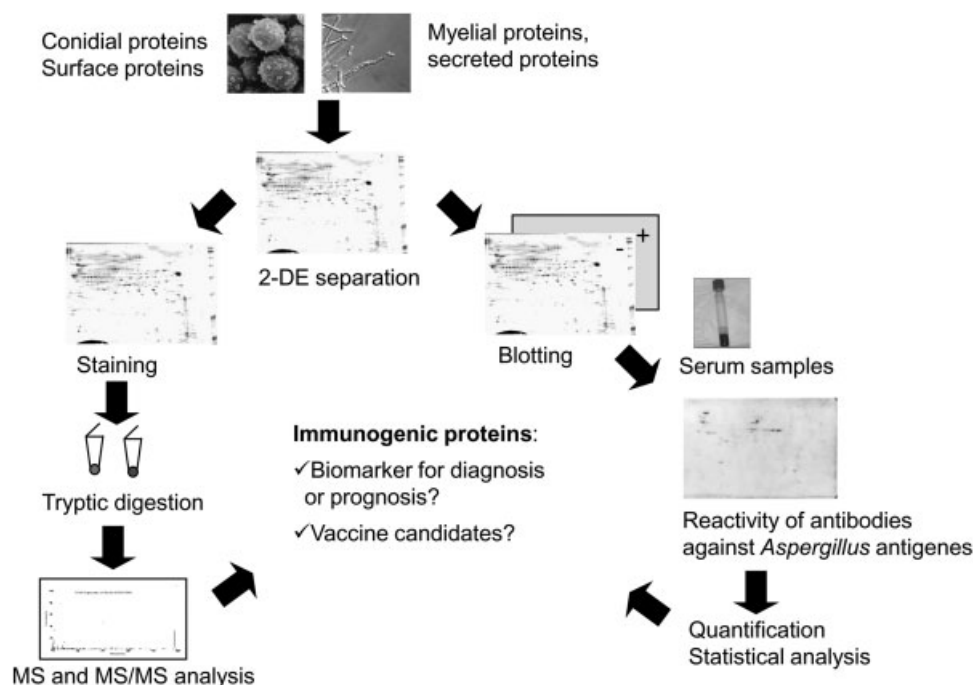


Figure 1. Workflow for the detection of *Aspergillus* antigens by a combination of 2-DE and immunoblotting.

[101]. A majority of the allergenic proteins were classified as proteins involved in metabolism, carbohydrate transport, energy conservation and stress response. Interestingly, some proteins showed strain-specific immunoreactivity and were only detected in one of the two *A. fumigatus* strains used in this study. In another study, a rabbit model of IA was used to screen for *A. fumigatus* antigens with an ability to elicit a protective immune response and here also, new immunoreactive proteins were found [102].

5.2 Drug response

Because of the rising number of severe fungal infections and the development of drug resistance, novel antifungal drugs are urgently needed. To identify new targets and to elucidate the molecular interactions of antifungal molecules with the fungal cell, proteomic approaches are helpful tools. Both the response of *A. fumigatus* to established antifungal drugs and to new antifungal agents were characterised by 2-DE and gel-free proteomic approaches [48, 103–106]. An initial study of Melin et al. [103] used 2-DE to analyse the effect of the antibiotic compound concanamycin A, a natural product of the Gram-positive bacterium *Streptomyces halstedii*, on the protein expression of *A. nidulans*. A substantial number of proteins were either up- or downregulated: One of the proteins that decreased in abundance, CpcB, is known to be involved in the initiation of sexual development and the response to amino acid starvation. Gautam et al. [106] studied the antifungal action of the drug amphotericin B (AMB) and identified 48 proteins affected by continuous exposure to amphotericin B, which are involved in various metabolic

processes like the ergosterol and heme biosynthesis pathway, the cell wall maintenance (downregulation of the hydrophobin RodB), oxidative stress (upregulation of Mn-superoxide dismutases and catalases), transporter proteins as well as primary metabolism. In comparison to that, the analysis of the response of *A. fumigatus* to the cell wall-targeting antifungal drug caspofungin led to a completely different picture. Many ribosomal proteins were shown to be increasing in the presence of caspofungin. By contrast, the mitochondrial hypoxia-responsive domain protein was downregulated more than 16-fold and was suggested to serve as potential biomarker for caspofungin-treated cells [48].

Besides the development of new therapeutic procedures, the improvement of the diagnosis of IA is of uttermost importance to achieve a better therapeutic outcome. Gonzales et al. [107] applied a SELDI-MS approach to discover biomarkers that could distinguish experimental IA from *Pseudomonas pneumonia* lung infections. Indeed, at later stages of infection, unique protein profiles, especially differently expressed acute-phase reactants, were detected in the blood of infected rabbits. In the next step, these experimental data have to be verified with clinical samples.

6 Concluding remarks

Until now, proteomic surveys in comparison to transcriptomic and genetic studies are under-represented in the area of *Aspergillus* research. However, a lot of progress has been made meanwhile and exciting insights into the *Aspergillus* physiology, biotechnological potential and pathogenicity have been gained. The application of new

proteomic technologies will definitely accelerate the growth of knowledge on the biology of *Aspergillus* sp., e.g. the use of high-resolution MS, targeted proteomic techniques like multi-reaction monitoring (MRM) and the employment of improved identification and quantification algorithms.

One of the major challenges for the future will be to make use of the mass of information that is being collected by transcriptome and proteome studies. Thus, an integrative data warehouse is needed, which provides besides sequence data also additional information about the regulation of a gene and protein, the condition when it is repressed or activated and the phenotype of the corresponding deletion mutant. Only stand-alone solutions for transcriptome and proteome data of pathogenic fungi [108] and for gene and protein sequence information from sequenced *Aspergillus* genomes have been established [109, 110]. An integrated data warehouse as a basis for a systems biology of *Aspergillus* sp. would be highly desirable.

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