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Proteomics of filamentous fungi

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Proteomic analysis, defined here as the global assessment of cellular proteins expressed in a particular biological state, is a powerful tool that can provide a systematic understanding of events at the molecular level. Proteomic studies of filamentous fungi have only recently begun to appear in the literature, despite the prevalence of these organisms in the biotechnology industry, and their importance as both human and plant pathogens. Here, we review recent publications that have used a proteomic approach to develop a better understanding of filamentous fungi, highlighting sample preparation methods and whole-cell cytoplasmic proteomics, as well as subproteomics of cell envelope, mitochondrial and secreted proteins.

Introduction

Filamentous fungi comprise an important class of organisms of significant commercial relevance, even though they typically receive less attention than their lower eukaryotic relatives, such as yeasts. For example, in the biotechnology industry, filamentous fungi are used to produce a wide variety of products ranging from human therapeutics (e.g. antibacterial and antifungal agents) to specialty chemicals (e.g. commercial enzymes, organic acids), which together represent billions of dollars in annual sales [1]. Just one class of compounds, the cholesterol-lowering statins, represents a market of almost US$15 billion per year in the USA [2,3]. Filamentous fungi are also notorious pathogens in both humans [4,5] and plants [6], and recently have received much public interest in the USA and in Denmark owing, respectively, to their prevalent infestation in buildings affected by Hurricane Katrina [7], and in schools affected by repeated flooding, raising health concerns for both adults and school children [8,9].

The importance of studying fungi can also be highlighted by the increasing number of genomes that have been sequenced. To date, 18 different species have been sequenced and annotated: *Aspergillus clavatus* [10], *A. flavus* [11,12], *A. fumigatus* [11,13], *A. nidulans* [13], *A. niger* [14], *A. oryzae* [12,13,15], *A. terreus* [10], *Botrytis cinerea* [16], *Chaetomium globosum* [16], *Coprinus cinereus* [16], *Fusarium graminearum* [16], *F. verticillioides* [17], *Magnaporthe grisea* [18], *Neurospora crassa* [19,20], *Phanerochaete chrysosporium* [21], *Rhizopus oryzae* [16,22], *Sclerotinia sclerotiorum* [16], and *Stagonospora nodorum* [16] (for reviews of fungal genomes, see [23–25] and for other on-going projects, see http://www.broad.mit.edu/annotation/fgi/). Yet despite their importance and the availability of sequenced genomes, there have been relatively few (although increasing) studies (Figure 1) on filamentous fungi compared with their simpler relatives, such as the model yeast *Saccharomyces cerevisiae* or the pathogen *Candida albicans*. This is true for both transcriptomic and proteomic analyses. We note that protein-level analysis is particularly relevant in eukaryotic systems, such as fungi because it allows location-specific analysis (i.e. subproteome, see Glossary), as well as the study of post-translational modifications (e.g. phosphorylation, glycosylation), which might impact on phenomena such as signal transduction [26].

In two previous reviews [27,28], it was noted that efforts toward post-genomic studies were just beginning in filamentous fungi, and to harness their potential as hosts for recombinant protein expression would require an increase in both transcript and proteomic related research. The earliest post-genomic studies of filamentous fungi were published at the beginning of the twenty-first century by Lim *et al.* [29] on *Trichoderma reesei* cell envelope proteins

Glossary

2DE: two-dimensional electrophoresis; gel-based separation of proteins by the orthogonal properties of isoelectric point (see isoelectric focusing) and molecular weight.
Isoelectric focusing: first dimension of separation in 2DE in which proteins are separated by their isoelectric point (pI); proteins are typically separated electrophoretically in gels containing an immobilized pH gradient (IPG).
LC-MS/MS: liquid chromatography-mass spectrometry/mass spectrometry, also called tandem mass spectrometry. A protein is hydrolyzed into peptides, which are separated by liquid chromatography, passed through an initial mass spectrometer to assess abundance, and subsequently fragmented via collision induced dissociation (CID). Fragment masses are assessed in the second mass spectrometer and used to reconstruct the original peptide sequence.
MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; one of the most common types of mass spectrometry used to perform PMF (see peptide mass fingerprinting). Either whole or fragmented proteins are ionized by a laser in the presence of crystallized matrix and are subsequently subjected to an electrical field to measure the amount of time that it takes for the particles to travel a known distance to correlate the time-of-flight of a particle with its mass-to-charge ratio.
Peptide mass fingerprinting (PMF): method of identifying unknown proteins by hydrolyzing them with specific proteases (e.g. trypsin) to generate peptides whose masses are determined by mass spectrometry (usually MALDI-TOF). Identification is achieved through comparison with theoretical peptide masses from a protein sequence database.
Proteome: global set of proteins expressed in a cell at a given time and biological state.
SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; electrophoretic technique used to separate proteins according to their molecular weight.
Subproteome: proteome of a defined subset of an organism, primarily specific organelles.
Secretome: proteome of the secreted proteins and the cellular machinery involved in their secretion.
Transcriptome: set of transcripts (i.e. mRNAs) expressed in a cell's particular physiological state; assessed via microarrays; mRNA complement of the proteome.

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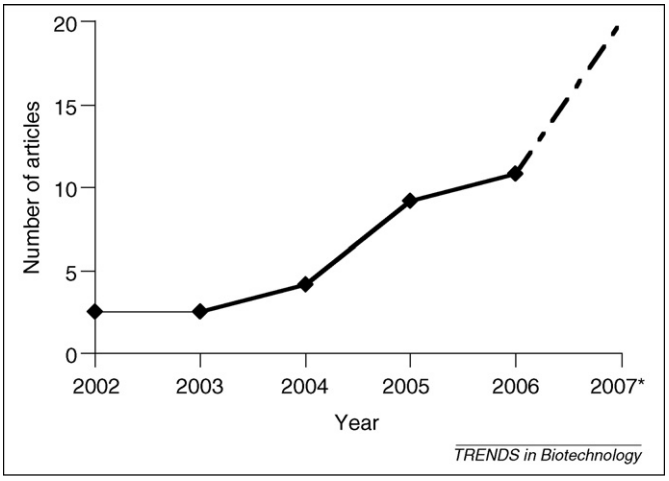

Figure 1. The number of new articles (including reviews) that have appeared in each of the past five years related to filamentous fungal proteomics. *Represents a projection as of April 2007.

and by Bruneau *et al.* [30] on *A. fumigatus* glycosylphosphatidylinositol-anchored proteins. Since then, a significant number of post-genomic studies have been published ([31], Table 1), and we believe the filamentous fungal research community has now moved beyond its initial stage into a posture of active research. Several reviews have addressed post-genomic fungal studies from a general perspective [27,28,31–37], and a review of transcript analysis studies has just appeared [31]. Our goal here is to complement these previous publications and provide a survey of recent proteomic studies in filamentous fungi. Specifically, we report on publications from the past five years that relate to whole-cell cytoplasmic proteomics, subproteomics of cell envelope proteins and of mitochondrial proteins, and the secretome of filamentous fungi. We have excluded studies of dimorphic fungi (e.g. *C. albicans*, *Ustilago maydis*) and yeast (for a recent review on *S. cerevisiae* proteomics, see [38]) to further narrow our scope.

Table 1. List of filamentous fungal proteomics papers

Category or species	Refs
Review	[27,28,32–37]
Aspergilli	
Sample preparation	[41,44,50,65]
Intracellular proteomics	[40,43,50,57,58]
Membrane subproteomics	[30,61]
Secretomics	[65–68]
Botrytis cinerea	
Intracellular proteomics	[51,59]
Neurospora crassa	
Mitochondrial subproteomics	[62]
Penicillium expansum	
Secretomics	[75]
Phanerochaete chrysosporium	
Intracellular proteomics	[46]
Membrane subproteomics	[46]
Mitochondrial subproteomics	[48]
Secretomics	[72–74]
Pleurotus sapidus	
Secretomics	[71]
Sclerotinia sclerotiorum	
Secretomics	[49]
Terebralia palustris	
Sample preparation	[45]
Trichoderma	
Sample preparation	[53]
Intracellular proteomics	[54]
Membrane subproteomics	[29]
Mitochondrial subproteomics	[42]
Proteasome subproteomics	[63]
Secretomics	[47,69,70]

Cell wall lysis and sample preparation

Because filamentous fungi have an exceptionally strong cell wall [39], several early studies were devoted to overcoming this challenge by providing an effective means of cell lysis for adequate release of intracellular proteins. For example, several researchers [40–43] used mechanical lysis via glass beads to liberate cytoplasmic protein, and this approach has been more efficient than either chemical or enzymatic extraction methods [44]. In an alternative approach, Shimizu and Wariishi [45] bypassed the difficulty of lysing the fungal cell wall by generating protoplasts of *Tyromyces palustris*. Two-dimensional electrophoresis (2DE) patterns from protoplasts were better visualized than proteins obtained from disrupting the fungal cell wall using SDS extraction. Since then, however, the most widely used method of extraction seems to be grinding in liquid nitrogen using a mortar and pestle [45–51].

Once the cells are lysed, the protein solution is often purified via trichloroacetic acid (TCA) precipitation to remove contaminants that can be problematic during isoelectric focusing [52]. Although effective in cleaning the sample, TCA-treatment makes subsequent protein solubilization for isoelectric focusing difficult. To circumvent this challenge, Nandakumar *et al.* [41] developed a time-saving protocol involving a brief treatment with sodium hydroxide to improve solubilization of TCA-precipitated proteins. More recently, Kniemeyer *et al.* [50] further optimized precipitate resolubilization with zwitterionic detergents. Others have reported an improvement by using a phosphate buffer solubilization before the precipitation [51], as well as the use of acidic extraction solution to reduce streaking of fungal samples caused by their cell wall [53].

Intracellular proteomics

One of the earliest intracellular filamentous fungal proteomic studies was performed by Hernández-Macedo *et al.* [46] on the wood-degrading fungi *P. chrysosporium* and *Lentinula edodes*. Using 2DE to conduct a differential comparison of cytoplasmic protein expression patterns in the presence or absence of iron, they visualized 21 proteins related to iron uptake in these ligninolytic fungi. However, the subsequent identification of these proteins was deficient and therefore Grinyer *et al.* [54] provided further progress in fungal proteomics by using mass spectrometry (both MALDI-TOF and LC-MS/MS) to identify proteins from *T. harzianum* whole-cell protein extract. Of the hundreds of proteins resolved in a single gel, the researchers identified 25 (out of 96 attempted) to provide an initial proteome map. Although this identification approach has been commonly used in proteomic studies of other organisms [55], Grinyer *et al.* were the first to use it to study filamentous fungi. Building on this established approach,

the researchers further studied the differential whole-cell proteome, as well as the secretome (proteome of secreted proteins), of *T. atroviride* grown in media containing either *Rhizoctonia solani* cell walls or glucose [47]. The researchers identified 24 protein spots, which contained both previously known cell wall-degrading enzymes and previously uncharacterized novel proteases.

Several proteomic studies have begun to appear in the literature for the genus *Aspergillus*. Petter Melin and colleagues provided the first protein identifications for the *A. nidulans* proteome [40,43]: their more recent report is particularly interesting. They co-cultivated the fungus with a lactic acid-producing bacteria, and showed specific changes in protein expression levels that correlated with the morphological changes caused by the co-cultivation. Two other reports characterized the proteome of *A. fumigatus,* perhaps the most prominent filamentous fungal human pathogen [56]. The first *A. fumigatus* proteome map was provided by Kniemeyer *et al.* [50] who conducted a systematic characterization of carbon catabolite repression by comparing protein expression patterns during growth on two different carbon sources. For growth on ethanol, 52 proteins were identified, for which many key gluconeogenesis, glyoxylate cycle, and ethanol degradation enzymes were found to be up-regulated. Later, Carberry *et al.* [57] added 28 additional protein identifications to the *A. fumigatus* proteome map, showing for the first time that the eukaryotic elongation factor 1Bγ protein exhibits glutathione transferase activity. This latter example illustrates that proteomic analysis not only provides a systematic perspective on fungal physiology, but also that it serves as a hypotheses-generating tool. We have observed a similar benefit when we recently updated the *A. nidulans* proteome map with identification of 30 additional proteins, including five that were not characterized and that are involved in osmoadaptation [58].

Proteomic analysis is also being used to develop systematic understanding of virulence factors in pathogenic fungi. For example, Fernández-Acero *et al.* [51], who provided the first proteome 2DE map of *Botrytis cinerea*, found that many of the identified proteins were isoforms of malate dehydrogenase and glyceraldehyde-3-phosphate dehydrogenase, correlating them to the phytopathogenic nature of *B. cinerea*. The authors followed up their first study by identifying pathogenicity factors and therapeutic targets for *B. cinerea* [59]. Similarly, the proteome of another phytopathogenic fungus, *S. sclerotiorum*, was recently mapped [49] and provided clues that α-L-arabinofuranosidase might be involved in the pathogenicity of the fungus.

Proteomic analysis in fungi is also providing insight related to systematic metabolic flux changes. Shimizu *et al.* [48] resolved 1100 intracellular and 300 mitochondrial proteins of *P. chrysosporium* in 2D gels and observed that 47 intracellular, and 10 mitochondrial proteins were differentially expressed when grown in the presence of vanillin. Their study not only identified key enzymes involved in vanillin metabolism, but also showed *P. chrysosporium*'s metabolic shift from glyoxylate cycle to the tricarboxylic acid cycle. Similarly, our work [58] showed that *A. nidulans* shifts metabolic flux toward glycerol biosynthesis during osmoadaptation, and has reduced expression of pathways that are downstream to the tricarboxylic acid cycle (e.g. lysine biosynthesis), and potentially has an increased protein turnover, as evidenced by increased expression of heat shock proteins and Shp1-like protein degradation protein. These studies demonstrate the capacity of proteomics to characterize systematically the various biochemical pathways that might be involved in adaptation to changing environments.

Subproteomics

We use the term 'subproteomics' to describe proteomic analysis of a defined subset of an organism's protein complement, primarily specific organelles [60]. Hernández-Macedo *et al.* [46] described procedures for plasma membrane and outer membrane protein extraction of *P. chrysosporium* and *L. edodes*, although the proteins were only visualized in one-dimensional SDS–PAGE rather than 2DE. Later, Asif *et al.* [61] provided the first subproteome map of *A. fumigatus* surface proteins, with the goal of finding potential therapeutic targets against this human pathogen. It is likely that future cell wall and membrane subproteomics studies will provide a systematic understanding of proteins involved in both protein secretion and in cell-to-cell interaction during pathogenesis.

Mitochondria have also received attention. Grinyer *et al.* [42] were the first to publish a mitochondrial subproteome, describing a successful sample preparation protocol and mitochondrial proteome map for *T. harzianum*. Based on protein databases of *N. crassa, A. nidulans, A. oryzae*, *S. cerevisiae*, and *Schizosaccharomyces pombe*, they identified 25 unique mitochondrial proteins involved in the tricarboxylic acid cycle, chaperones, protein-binding and transport proteins, as well as mitochondrial integral membrane proteins. More recently, Schmitt *et al.* [62] reported on proteomic analysis of the mitochondrial outer membrane of *N. crassa*. The researchers employed LC-MS/MS and MALDI-TOF from 1-D SDS–PAGE to circumvent the difficulty of solubilizing and focusing hydrophobic mitochondrial membrane proteins for 2DE. They were able to identify 30 proteins, of which some are known to be involved in transport (import machinery and transporters) and overall mitochondrial morphology. Most recently, Grinyer *et al.* [63] separated and identified 13 of the 14 subunits of the *T. reesei* 20S proteasome, providing the first filamentous fungal proteasome proteomics.

These reports imply that systematic, whole- and even sub-organelle proteomics is possible once adequate organelle separation protocols are in place. The advantage of a subproteomic approach is that it enables the protein expression to be localized in a particular organelle, thereby providing additional insight into the function of the protein in the given physiological state of the cell.

Secretome

The secretome has been defined as the combination of native secreted proteins and the cellular machinery involved in their secretion [64]. Secretome-related studies are particularly relevant in understanding filamentous fungi because many fungi secrete a vast number of proteins

to accommodate their saprotrophic lifestyle. In light of this, it has been said that unlike animals, 'fungi digest their food and [then] 'eat' it' [7], illustrating the large number of extracellular hydrolytic enzymes necessary to digest a plethora of potential substrates. Many of these proteins are of special interest in the study of pathogens [49,57,59] or during production of recombinant proteins in the biotechnology industry [28]. As a result, a significant number of publications have described the fungal secretome. This might also be owing to the fact that secretome sample preparation is much faster and simpler than extraction and preparation of intracellular proteins.

Wilson Francisco and colleagues provided pioneering contributions to this field, establishing a sample preparation protocol for the fungal secretome [65]. Using this protocol, they studied *A. flavus* and identified 22 secreted proteins involved in rutin degradation [66]. This helped develop an initial understanding of the enzymes involved in degradation of secondary metabolites for cellular consumption. They continued this work using LC-MS/MS to identify an additional 51 secreted proteins, of which 18 were found to be in the rutin degradation pathway [67]. Oda *et al.* [68] studied the secretome of *A. oryzae* and identified 29 extracellular proteins when fungi were grown in either liquid or solid-state culture. Several of the identified proteins were sequestered in cell walls during liquid culture but passed through the cell wall during solid-state growth.

Suárez *et al.* [69] studied the secretome of *T. harzianum* grown using either chitin (a key cell wall component) or the actual cell walls of other fungi (*R. solani, B. cinerea,* or *Pythium ultimum*) as a nutrient source. For each different substrate, they found significant differences in 2DE maps of extracellular proteins. However, despite these differences, the most abundant protein under all conditions was a novel aspartic protease (P6281), which showed strong homology with polyporopepsin from *Irpex lacteus.* This led to speculation that this protein has a fundamental role in the parasitic activity of *Trichoderma* spp. Similarly, Marra *et al.* [70] provided a novel proteomic study of three-way interaction between *T. atroviride, R. solani, B. cinerea.* They identified numerous proteins involved in multiple-species cross-talk, providing insight to the host-pathogen interaction in nature, as well as to proteins that are potentially specific to pathogenesis. A similar approach was used by Zorn *et al.* [71], in which the secretome of *Pleurotus sapidus* grown on peanut shells was observed. The researchers found that most secreted proteins had acidic isoelectric points (pIs) and were various metallopeptidases and serine proteases. In an alternative, completely non-gel based approach, Dan Cullen and colleagues provided a comprehensive identification of the *P. chrysosporium* secretome using a combination of shotgun LC-MS/MS and database prediction [72,73] (see also review in [74]).

Concluding remarks

It is our opinion that the field of fungal proteomics is rapidly entering an 'exponential' phase as evidenced by an apparent increase in the rate of relevant publications (Figure 1). This observation is supported by an increased number of presentations that appeared at recent conferences on both sides of the Atlantic, such as the 24th Fungal Genetics Conference at Asilomar (2007), the 8th European Conference on Fungal Genetics at Vienna (2006), and the Second International Fungal Proteomics Symposium at Baltimore (2006). The increasing number of available genomes of filamentous fungi will also aid this acceleration of publications and we eagerly anticipate many publications on fungal proteomics that will emerge in the near future.

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