Advances in fungal proteomics

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Summary
Proteomics, the global analysis of proteins, will contribute greatly to our understanding of gene function in the post-genomic era. This review summarizes recent developments in fungal proteomics and also generalizes protocols for sample preparation from plant pathogenic fungi. Challenges and future perspectives of proteomics are discussed as well.

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Introduction
Proteomics is defined as the systematic analysis of the proteome, the protein complement expressed by a genome, cell, tissue or organism. This technology allows qualitative and quantitative measurements of large numbers of proteins that directly influence cellular biochemistry, and thus provide accurate analysis of cellular state or system changes during growth, development and response to environmental factors. It is a key technology for the study of highly complex and dynamic biological systems (Chen and Harmon, 2006).

Sequencing of over 25 fungal genomes has been completed (http://www.broad.mit.edu). Now a major challenge in modern fungal biology is to understand the expression, function and regulation of the entire set of proteins encoded by fungal genomes. This is the aim of newly emerging field of proteomics, i.e., fungal proteomics. This information will be invaluable for understanding plant–fungal interactions, pathogenesis and fungal colonization. Proteomics complements other functional genomics approaches including transcriptomics. Transcriptomics is the study of global change of gene expression at mRNA level. But proteomics is a more reliable technique than transcriptomics because proteins are directly related to function (or...
phenotype). Recently, Tong et al. (2001) developed a method to produce a global map of gene function. They assembled an ordered array of around 4700 viable yeast gene-deletion mutants and developed a series of pinning procedures in which mating and meiotic recombination are used to generate haploid double mutants. A query mutation is first introduced into a haploid starting strain, of mating type MAT\textsuperscript{x}, and then crossed to the array of gene-deletion mutants of the opposite mating type, MAT\textsuperscript{a}. Sporulation of resultant diploid cells leads to the formation of double-mutant meiotic progeny. The MAT\textsuperscript{x} starting strain carries a reporter, MFA1pr-HIS3, that is only expressed in MAT\textsuperscript{a} cells and allows for germination of MAT\textsuperscript{a} meiotic progeny, which ensures that carryover of the diploid parental strain and/or conjugation of meiotic progeny does not give rise to false-negative interactions. Both the query mutation and the gene-deletion mutations were linked to dominant selectable markers to allow for selection of double mutants. Final pinning results in an ordered array of double-mutant haploid strains whose growth rate is monitored by visual inspection or image analysis of colony size. This method is termed as synthetic genetic array (SGA). But SGA is a proteomics-dependent procedure.

Proteomics studies have already been started in mycoparasitic fungi like Trichoderma harzianum (Grinyer et al., 2005) Trichoderma atroviride (Grinyer et al., 2004) that could provide an insight into understanding mechanism involved in biological control of pathogenic fungi. Even though, fungal proteomics is in its infant stage. This article provides an overview of fungal proteomics, its advancements and challenges.

**Sample preparation**

Since most fungi possess an exceptionally robust cell wall, effective extraction of proteins is a key step for fungal proteomic studies. For total protein extraction, an ideal protocol would reproducibly capture all the protein species in a proteome with low contamination of other molecules. A slightly modified protein extraction method from rice blast fungus, Magnaporthe grisea (Kim et al., 2004) provides reproducible presentation of total fungal proteins on two-dimensional (2D) gels to some extent. In this method, proteins are extracted using Mg/CHAPS extraction buffer containing 0.5 M Tris–HCl pH 8.3, 2% CHAPS, 20 mM MgCl\textsubscript{2}, 20 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). A most widely used protocol for protein precipitation is using 10% trichloroacetic acid (TCA) in acetone with 0.07% 2-mercaptoethanol or 20 mM DTT. It is based on protein denaturation under acidic and/or hydrophobic conditions that help to concentrate proteins and remove contaminants. The combination of TCA and acetone is usually more effective than either TCA or acetone alone.

Since the cell wall is thought to cause ineffective protein extraction from basidiomycetous cells, the utilization of fungal protoplast is of great interest. Shimizu and Wariishi (2005) used protoplast from Tyromyces palustris mycelial cells to extract intracellular proteins. Proteins were isolated using sodium dodecyl sulfate (SDS) buffer containing 4% SDS, 2% DTT, 20% glycerol, 20 mM PMSF and 100 mM Tris–HCl (pH 7.4). Intracellular proteins were precipitated with four volumes of cold acetone. However, due to the diversity of protein abundance, molecular weight, charge, hydrophobicity, post-translational processing and modifications, and complexation with other molecules, no single extraction protocol is effective.

In order to enhance the coverage and detection of subsets of proteome such as membrane proteins and low-abundant proteins, various strategies have been developed over the years to fractionate proteins into subproteomes based on biochemical, biophysical and cellular properties. These front-end fractionation procedures have greatly improved detection and resolution by reducing the overall sample complexity, and thus increases proteome coverage, e.g., the detection of low-abundant proteins.

**Proteome profiling, MS tools and database search**

Elucidating how the proteome changes in response to biotic stress like fungal invasion is crucial to understand the molecular mechanisms underlying plant-pathogen interaction and pathogenesis. Recent years have witnessed a revolution in the development of new approaches for identifying large numbers of proteins expressed in cells and also for globally detecting the differences in levels of proteins in different cell states. The principles of fungal proteomics are outlined in Fig. 1.

**Electrophoresis**

For several decades, one-dimensional (1D) SDS-polyacrylamide gel electrophoresis (PAGE) has been one of the most widely used tools for the separation of total protein extracts as well as protein fractions obtained from various prefractionation procedures.
Currently, two gel-based [two-dimensional electrophoresis (2DE) and two-dimensional fluorescence difference gel electrophoresis (2D-DIGE)] and one non-gel-based (multidimensional liquid chromatography) protein separation techniques are widely used in fungal proteomics. 2D gel electrophoresis has been the primary tool for obtaining a global picture of the expression profile of a proteome under various conditions. In this method, proteins are first separated in one direction by isoelectric focusing and then in the orthogonal direction by molecular mass using electrophoresis in a slab gel containing SDS-PAGE (O’Farrell et al., 1997). Using this approach, several thousand protein species can be resolved in a single slab gel. 2D-DIGE enables to perform high-throughput, differential protein expression analysis to compare directly, on a single gel, the differences in protein expression levels between different complex protein samples. The
main advantage of 2D-DIGE on 2DE is its unrivaled performance, attributable to a unique experimental design in which each protein spot on the gel is represented by its own internal standard. The standardization made possible by direct in-gel comparisons of as many as three samples, allows greater reproducibility of results, savings of time and resources, and increased analytical power.

But reproducibility and resolution are still remained challenges for two gel based protein separation techniques. Multidimensional protein identification technology (MudPIT) is an unbiased method as compared to traditional 2D electrophoresis. In this method, multidimensional liquid chromatography is coupled with tandem mass spectrometry (MS), and database searching by the SEQUEST algorithm. MudPIT was first applied to the fungal proteome of the Saccharomyces cerevisiae strain BJ5460 grown to mid-log phase and yielded the largest proteome analysis to date. A total of 1484 proteins were detected and identified. Categorization of these hits demonstrated the ability of this technology to detect and identify proteins rarely seen in proteome analysis, including low-abundance proteins like transcription factors and protein kinases (Washburn et al., 2001).

**MS tools**

In recent years, protein separation methods coupled with various MS technologies have evolved as the dominant tools in the field of protein identification and protein complex deconvolution (Figeys et al., 2001). The key developments were the invention of the time-of-flight (TOF) MS and relatively non-destructive methods to convert proteins into volatile ions. A typical MS consists of an ion source, a mass analyzer and a detector. Two “soft ionization” methods, namely matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), have made it possible to generate and ionize large biomolecules, such as peptides and proteins (Yates, 1998; Godovac-Zimmermann and Brown, 2001; Mann et al., 2001). There are four types of mass analyzers currently used in proteomics research. They are ion trap, TOF quadrupole and Fourier transform ion cyclotron (FT-MS). MALDI is usually coupled to TOF analyzers that measure the mass of intact peptides. More recently, new configurations of ion sources and mass analyzers have found wide application for protein analysis. A new matrix-assisted laser-desorption/ionization TOF/TOF MS with the novel laser-induced fragmentation technique (MALDI LiFT-TOF/TOF MS) provides high sensitivity (atto-mole range) for peptide mass fingerprints (PMF). It is also possible to analyze fragment ions generated by any one of three different modes of dissociation: laser-induced dissociation and high-energy collision-induced dissociation (CID) as real MS/MS techniques and in-source decay in the reflector mode of the mass analyzer (reISD) as a pseudo-MS/MS technique. ESI has mostly been coupled to ion traps and triple quadrupole or hybrid TOF MS and used to generate fragments ion spectra (CID spectra) of selected precursor ions. Compared with MALDI, ESI has a significant advantage in the ease of coupling to separation techniques such as LC and high-performance liquid chromatography (HPLC), allowing high throughput and on-line analysis of peptide or protein mixtures (Ducret et al., 1998; Gatlin et al., 1998). Typically, a mixture of proteins is first separated by LC followed by tandem MS (MS/MS). In this procedure, a mixture of charged peptides is separated in the first MS according to their m/z ratios to create a list of the most intense peptide peaks. In the second MS analysis, the instrument is adjusted so that only a specific m/z species is directed into a collision cell to generate “daughter” ions derived from the “parent” species. LC-MS has been applied to large-scale protein characterization and identification (Zhu et al., 2003). The Yates (1998) group was able to resolve and identify 1484 proteins from yeast in a single experiment. Unlike the 2D/MS approaches, the authors demonstrated that even low-abundance proteins could be clearly identified, such as certain protein kinases. In addition, 131 of the proteins identified have three or more predicted transmembrane domains, suggesting that this approach was able to readily detect membrane proteins.

**Database search**

Peptide mass fingerprints (PMF) are utilized for protein identification by analyzing the sizes of tryptic fragments via the MASCOT (http://www.matrixscience) search engine using the entire NCBI fungal protein database. For effective PMF analysis, it is assumed that peptides should be monoisotopic and the possibility for the oxidation of methionine residues is considered. The fingerprinting method allows for a maximum of one missed tryptic cleavage per protein. The maximum deviation permitted for matching the peptide mass values is 100 ppm. Scores greater than 65 are considered to be significant (p = 0.005).

MS/MS spectra are searched against a composite database containing the translated genome sequences of S. cerevisiae and Escherichia coli, known fungal

**Post-translational modifications (PTMs)**

Covalent modifications to protein structures, which occur either co- or post-translationally, play a pivotal role in regulating protein activity. Identification of the type of modification and its location often provide crucial information for understanding the function or regulation of a given protein in biological pathways. To date, over 300 PTMs are known. Phosphorylation, glycosylation, sulfation, acetylation, myristoylation, palmitoylation, methylation, prenylation and ubiquitylation as well as many other modifications are extremely important for protein function as they can determine activity, stability, localization and turnover. Many of the PTMs are regulatory and reversible, most notably protein phosphorylation, which controls biological function through a multitude of mechanisms. These modifications are not generally apparent from genomic sequence or mRNA expression data.

The first step in PTMs studies is the identification of the protein to be studied. Protein modifications are typically not homogeneous. 2D electrophoresis often has sufficient resolution to separate the modification states of a protein directly. For example, phosphorylation changes the protein charge and is often indicated by a horizontal trail of protein spots on the 2D gels. It is often advantageous to reduce the complexity and to increase the amount of modified proteins available for analysis, proteins are often subjected to prefractionation such as chromatographic purification and affinity enrichment. Often, SDS-PAGE is the final preparation step to isolate at least Coomassie stainable amounts (~1 μg) to increase the chance of detecting and characterizing modifications in proteins. Isolated proteins are digested enzymatically or chemically degraded. The resulting peptides are usually separated by HPLC. In Edman degradation, collected peptides fractions are applied to the sequencer and their amino acid sequence determined. Modified amino acids become apparent because of their absence or retention time shift in the corresponding sequence cycle. If the mass of the intact peptide has been determined, then the nature of modification can be more confidently assessed. One method is to determine phosphorylation using a combination of MS and Edman degradation. Proteins are labeled with $^{32}$P, and the cycle in which the radiolabeled amino acid released, is recorded. Together with the mass of the peptide, this often allows the determination of the site of phosphorylation (Borner et al., 2003; Peltier et al., 2004). Thus, proteomics has played an important role in identifying and characterizing post-translational protein modifications.

**Protein localization**

Protein localization provides valuable information in elucidating eukaryotic protein function. To determine the subcellular localization of a protein, its corresponding gene is typically either fused to a reporter or tagged with an epitope. Reporters and epitope tags are fused routinely to either the N or C termini of target genes, a choice that can be critical in obtaining accurate localization data. Organellar-specific targeting signals (e.g., mitochondrial targeting peptides and nuclear localization signals) are often located at the N terminus (Silver, 1991); N-terminal reporter fusions may disrupt these sequences, resulting in anomalous protein localizations. In other cases, C-terminal sequences may be important for proper function and regulation, as recently shown from analysis of the yeast $\gamma$-tubulin-like protein Tub4p (Vogel et al., 2001). Using directed topoisomerase1-mediated cloning strategies and genome-wide transposon mutagenesis, 60% of the S. cerevisiae proteome was epitope-tagged. By high-throughput immunolocalization of tagged gene products, the subcellular localization of 2744 yeast proteins has been determined (Kumar et al., 2002). The discovery of green fluorescent protein (GFP) and the development of its spectral variants (Tsien, 1998) have opened the door to analysis of proteins in living cells using the light microscope. Large-scale approaches of localizing GFP-tagged proteins in cells have been performed in the genetically amenable yeast S. pombe (Sawin and Nurse, 1996; Ding et al., 2000).

**Protein interactions (cell map proteomics)**

Cell map proteomics is defined as the systematic analysis of protein-protein interaction through
isolation of protein complexes. It is widely acknowledged that proteins rarely act as single isolated species when performing their functions in vivo (Yanagida, 2002). Cellular functions emerge from the properties of dynamic interconnected protein networks. To understand the complex behavior of networks within cells, individual protein interactions must be analyzed in intact cells with high spatial and temporal resolution. Two approaches have been used to map protein–protein interaction: the yeast two-hybrid method and biochemical co-purification of complexes using affinity tags, coupled with protein identification using MS.

In yeast two-hybrid system, a component of interest (bait) is typically fused to a DNA-binding domain. Other proteins (preys), which are fused to a transcription-activating domain, are screened for physical interactions with the bait protein using the activation of a transcription reporter construct as the detection method (read out). To map protein interactions in *S. cerevisiae*, different genome-wide two-hybrid strategies have been used. One such approach involved screening a large number of individual proteins against a comprehensive library of randomly generated fragments, as was used to identify numerous interactions for proteins implicated in RNA splicing (Fromont-Racine et al., 2000). The second approach used systematic testing of every possible combination of proteins using a mating assay with a comprehensive array of strains. In this way, 192 baits were screened against an array of essentially all activation-domain fusions of full-length yeast ORFs to identify 281 putative interactions (Uetz et al., 2000). A third approach used a one-by-many mating strategy in which each member of a nearly complete set of strains expressing yeast ORFs as DNA-binding domain hybrids was mated to a library of strains containing activation-domain fusions of full-length yeast ORFs resulting in 629 positives (Uetz et al., 2000). A fourth variation involved mating of defined pools of strain arrays (Ito et al., 2001). This approach required cloning all of the yeast ORFs into both two-hybrid vectors, followed by pooling sets of 96 transformants each. Mating was conducted for the 62 × 62 combinations of pools, and positives were sequenced, resulting in a total of 4549 positives, of which the 841 that were identified more than three times form a core data set (Phizicky et al., 2003).

Another method for identifying protein–protein interactions is based on the “fluorescence resonance energy transfer”, which describes the energy transfer from an activated donor-fluorophore to an acceptor-fluorophore localized in close vicinity to each other. This phenomenon can be used to monitor molecular interactions, if both interaction partners are labeled with suitable fluorophores, as for example variants of the GFP. Fusion proteins containing GFP sequences may be recombinantly expressed and subsequently monitored in the intact cell. Thus interactions of different fusion proteins can easily be observed under physiological conditions. Dynamic studies on the formation of stable protein complexes and even of transient interactions become possible and complement biochemical interaction experiments.

Challenges and future perspectives

Ample progress has been made in the field of fungal proteomics in the past few years. It is due to the developments in sample preparation, high-resolution protein separation techniques, MS, MS software for effective protein identification and characterization, and bioinformatics technology. However there still exist different technical challenges. For example, there is no protein equivalent of PCR for amplification of low-abundance proteins, so a range of detection from one to several million molecules per cell is needed. Proteins have properties arising from their folded structures, so generic methods are difficult to design and apply, and the analysis and significance of PTMs provide a major challenge. Certain technological processes, particularly protein separation and analysis, are inherently skill-based and remain difficult to automate. Separation techniques such as DIGE may be more amenable to automation. But reproducibility is still a challenge in protein separation. However, many complementary technologies are being developed and either alone or in combination will undoubtedly assume prominent roles in the armouries of proteomics and functional or structural genomics-based approaches, whether in expression profiling or molecular interaction screening. These include protein arrays (Walter et al., 2000), the yeast two-hybrid system (Fromont-Racine et al., 2000), phage-display antibody libraries (Griffiths and Duncan, 1998), surface-enhanced laser desorption and ionization (Senior, 1999) and biological activity profiling of families of proteins such as proteases (McKerrow et al., 2000).

The proteome is dynamic, reflecting the conditions to which a cell is exposed or, for example, a specific disease process. There is therefore potentially a huge number of proteomes for each cell type. Hypothesis-driven research with careful selection of the specific features of a proteome that provide information relevant to the particular
biomedical question is particularly important given that the bottleneck is likely to lie not in identifying the proteins but in their downstream characterization.

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