

# The Application of Proteomic Techniques to Fungal Protein Identification and Quantification

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**ABSTRACT:** The number of sequenced genomes has increased rapidly in the last few years, supporting a revolution in bioinformatics that has been leveraged by scientists seeking to analyze the proteomes of numerous biological systems. The primary technique employed for the identification of peptides and proteins from biological sources is mass spectrometry (MS). This analytical process is usually in the form of whole-protein analysis (termed “top-down” proteomics) or analysis of enzymatically produced peptides (known as the “bottom-up” approach). This article will focus primarily on the more common bottom-up proteomics to include topics such as sample preparation, separation strategies, MS instrumentation, data analysis, and techniques for protein quantification. Strategies for preparation of samples for proteomic analysis, as well as tools for protein and peptide separation will be discussed. A general description of common MS instruments along with tandem mass spectrometry (MS/MS) will be given. Different methodologies of sample ionization including matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) will be discussed. Data analysis methods including database search algorithms and tools for protein sequence analysis will be introduced. We will also discuss experimental strategies for MS protein quantification using stable isotope labeling techniques and fluorescent labeling. We will introduce several fungal proteomic studies to illustrate the use of these methods. This article will allow investigators to gain a working knowledge of proteomics along with some strengths and weaknesses associated with the techniques presented.

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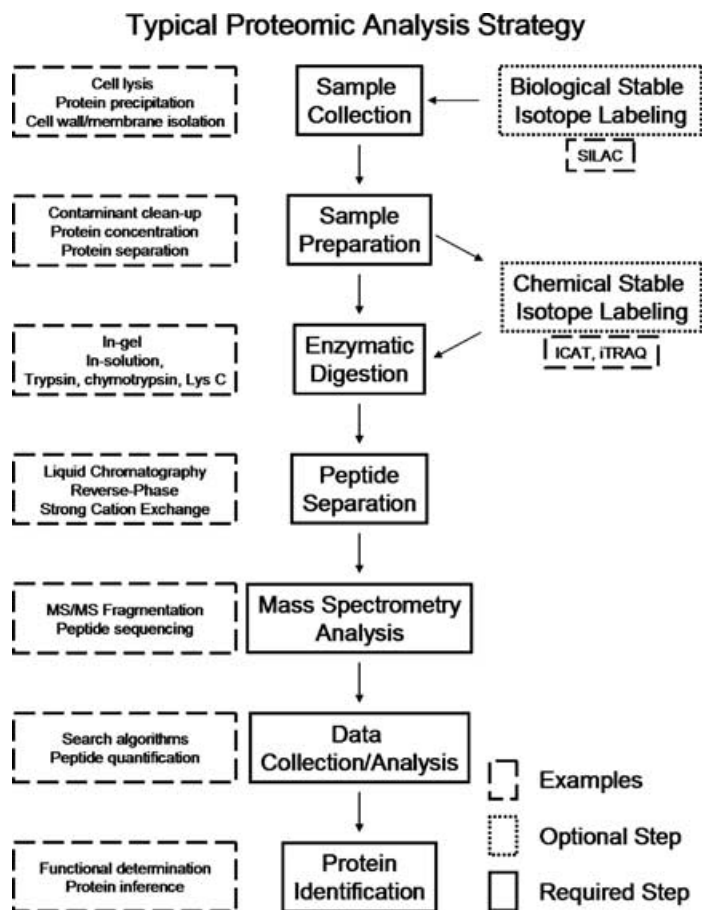
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## INTRODUCTION

With the increasing number of sequenced genomes, proteomics is a field of study that has expanded rapidly in the last decade to encompass a wide variety of techniques and technologies. While protein analysis is not new, many recent advances in bioinformatics as well as in mass spectrometry (MS) have increased the speed and breadth of samples that can be efficiently analyzed. Older methodologies, such as protein sequence determination by Edman degradation, amino acid composition analysis, or gel-based analytical methods like Western blots still have their place in specific research endeavors, but are somewhat less applicable to the high-throughput nature of modern proteomic analyses. This article is intended to be a brief overview of modern proteomics as it relates to the characterization and identification of fungal proteins. Readers desiring more comprehensive information can look to articles such as the 2003 review by Aebersold and Mann,<sup>1</sup> or more recent articles by Swanson and Washburn,<sup>2</sup> and Wysocki *et al.*<sup>3</sup>

A proteomic analysis of a system involves the collection, separation, identification, and functional determination of the expressed proteins of a sample,<sup>4</sup> which can lead to a better understanding of protein function and regulation of a system. A detailed analysis of the proteome can then lead to protein targets for disease identification, treatment, or vaccine development. The general steps of a proteomic analysis after the collection of a sample of interest are extraction of the proteins from the sample mixture, proteolytic digestion to produce peptides, peptide separation, peptide identification, and determination of identity and function of proteins present in the original sample. A brief overview of a typical proteomic analysis strategy is shown in FIGURE 1. The methods (sample preparation, ionization type, mass analyzer, etc.) employed for a proteomic analysis may vary depending on the starting material and the goal of the analysis.

There are many difficulties encountered in the analysis of proteins in a biological sample. The most obvious is the inherent complexity of many samples. Many proteins are present in locations that prohibit easy analysis, such as membrane-bound proteins which are difficult to solubilize. Very large and very small proteins can also be difficult to analyze and detect. Not all proteins are present in equal abundance, a concept known as dynamic range.<sup>5</sup> This leads to a difficulty in detection of low-abundance proteins in the presence of highly abundant ones. Unlike RNA-based methods of transcript amplification, sample protein levels cannot be increased to facilitate analysis of low-abundance proteins. Any undertaking of a proteomic analysis will likely require addressing at least one of these difficulties.



**FIGURE 1.** Typical proteomic analysis strategy. The typical strategy for a bottom-up proteomic analysis, including optional stable isotope labeling for protein quantification and examples of some of the techniques employed in each step.

Recent advances in transcript identification may lead investigators to use mRNA analysis to try to infer protein presence. It is important to note, however, that while analysis of the mRNA levels of a system provides insights into gene expression, those levels may not correlate with protein abundance. Protein and corresponding mRNA levels can vary dramatically. It is common for protein or mRNA levels to vary by as much as 30-fold with respect to each other, or in some cases for either to be absent.<sup>6</sup>

The most common methods of analysis use MS for protein and peptide identification. Sample proteins can then be analyzed whole, in what is known as a top-down proteomic analysis,<sup>7</sup> or analyzed as peptides from protein digestion in a bottom-up approach. Top-down proteomics is less popular,

primarily because of the need for expensive and complex high-resolution mass spectrometers. Bottom-up methods can include peptide identification by high-resolution mass determination from a single round of MS, known as peptide mass fingerprinting,<sup>8</sup> or more commonly, peptides are identified by peptide fragmentation in a tandem mass spectrometry experiment (MS/MS) to facilitate amino acid sequence correlation.<sup>9</sup> In MS/MS, the peptides are separated by the mass analyzer and then subjected to fragmentation. The masses of the fragment ions are determined by a second mass analyzer (or in a second round of MS in a trapping-type instrument). Since different peptides will fragment differently, this technique allows not only for the identification of peptides with different masses, but also those with the same or similar masses.<sup>10,11</sup>

## PROTEIN/PEPTIDE SEPARATION

Protein complexity can be reduced with some relatively simple methods of protein separation, such as one-<sup>12</sup> and two-dimensional<sup>13</sup> electrophoresis (1DE and 2DE). These gels are run under denaturing conditions, including heat, detergent (such as SDS), and a reductant (such as dithiothreitol [DTT] or  $\beta$ -mercaptoethanol) for disulfide bond cleavage. In addition to reducing the sample complexity, gel electrophoresis can also be used for sample cleanup (removal of salts, detergents, etc.). One-dimensional electrophoresis involves the separation of denatured proteins based on size, while 2DE starts with a separation of proteins by isoelectric point, followed by separation by size. A variation of the 2DE method using fluorescent dyes for protein quantification is known as difference gel electrophoresis (DIGE), which will be discussed later. After protein separation using electrophoresis, in-gel digestion is often used to produce and extract peptides prior to MS analysis.<sup>14</sup>

The complex peptide mixtures from a 1DE gel or solution digest in a bottom-up experiment can be further separated by using high-performance liquid chromatography (HPLC) online peptide separation methods. The most common peptide separation is known as reverse-phase (RP) LC. Using this method, the peptides are separated by hydrophobicity by eluting peptides bound to the RP packing material by means of an organic solvent (such as acetonitrile or methanol) gradient flow by HPLC.<sup>15</sup> Another LC separation method used is strong cation exchange (SCX), which separates peptides by charge, where the peptides bound to the SCX material are eluted by a salt gradient. SCX is often used in conjunction with RP separation in a method known as,<sup>16</sup> MultiDimensional Protein Identification Technology (MudPIT). MudPIT uses a column containing both RP and SCX chromatography phases described above, which allows for easier and automated analysis of biological mixtures, by reducing the complexity of peptides with a method that does not require protein separation by electrophoresis. While MudPIT is often used to analyze solution-digested

proteins, 1DE has been used as a sample clean-up step, followed by MudPIT analysis.

## IONIZATION METHODS

There are several types of ionization methods used for MS analysis, but there are two primary ones used in proteomics. Electrospray ionization (ESI),<sup>17</sup> and its closely-related small-volume cousin nanoelectrospray (nano-ESI),<sup>18</sup> involve injection of analyte (peptide or protein) molecules exiting the LC in solution into the mass spectrometer. A major advantage of ESI is the ease of coupling online separation methods, such as RP and SCX prior to ionization and MS analysis. Also, ESI produces multiply-charged ions, allowing for identification of larger ions in an instrument with a low mass-to-charge ( $m/z$ ) ratio limit.

The second major ionization method used in proteomics is matrix-assisted laser desorption/ionization (MALDI).<sup>19,20</sup> The sample of interest is mixed with a matrix, spotted onto a sample plate, and then excited by a laser beam, ionizing and transferring analyte molecules into the gas phase for analysis. Advantages of MALDI ionization include a larger analyte mass range and higher tolerance of salts than ESI.

## MASS ANALYZERS

After the protein or peptide molecules have been ionized and put into the gas phase, they enter the mass spectrometer for analysis. There are several methods of mass analysis in MS, but there are four major types used in proteomic analysis. All mass analyzers operate on the same basic principle of separation of ions by their  $m/z$  ratio. The first type is the quadrupole mass analyzer,<sup>21</sup> which is one of the oldest and best defined and has been a mainstay of MS analysis for decades. A major disadvantage associated with quadrupoles is the necessity of multiple mass analyzers to accomplish MS/MS.

There are two common ion-trap mass analyzers based on the physics of the quadrupole mass analyzer. The first is the quadrupole ion trap (QIT),<sup>22</sup> the second is the newer linear ion trap (LIT).<sup>23</sup> Another ion-trap mass analyzer is the Fourier transform ion cyclotron resonance (FTICR, or just FT)<sup>24</sup> MS that uses a superconducting magnet for ion control. The benefits of ion-trapping mass analyzers include the ability to perform multiple rounds of MS and prevent ion fragmentation within the same mass analyzer, as well as an increase in signal-to-noise ratio. Disadvantages of the QIT/LIT analyzers include limited resolution and mass accuracy. While the FT has excellent resolution and mass accuracy appropriate for top-down sequencing, it is relatively large and expensive and is more difficult to couple to LC-based sources.

The final common mass analyzer is the time of flight (TOF),<sup>25</sup> which is a much simpler system than either a quadrupole or FT-based mass analyzer. In

the TOF, ions are separated by the time it takes them to travel the length of the analyzer, with the smaller  $m/z$  ratio ions having an impact before larger ones. In addition to its simplicity of operation, the TOF mass analyzer is also valued for its enhanced mass accuracy and resolution over the quadrupole-based instruments. Disadvantages of TOF include the difficulty of coupling the pulsed analysis with continuous-ionization LC-based peptide separation, and the need for an additional mass analyzer to accomplish MS/MS. TOF instruments that are commonly used in proteomic analysis include the quadrupole coupled to a TOF (QTOF) or two TOF analyzers in sequence (TOF–TOF).

## DATA ANALYSIS

MS/MS spectra typically do not directly provide a peptide sequence. Instead, spectral information is matched to known peptide sequences from protein sequence databases. Some of the more popular database collections include the National Center for Biotechnology Information (NCBI), which includes most of the public domain sequence databases, included in the nonredundant (NR) database. Another collection of sequences is the Swiss Prot database, which has many sequences, but also has a large amount of annotation included with the sequences to allow for easier identification of functionality of listed proteins.<sup>11</sup> If the genome of the organism being analyzed has not been sequenced, the best strategy is to build a database of closely related species, or search against the NR database, with the realization that the larger the database, the longer the search process will take. There are several different sources for sequenced fungal genomes. Among these are the Broad Institute (<http://www.broad.mit.edu>), the Sanger Institute (<http://www.sanger.ac.uk>), The Institute for Genomic Research (TIGR) (<http://www.tigr.org>), and Génolevures (<http://cbi.labri.fr/Genolevures>). Peptide sequences are matched to spectral information using a database search algorithm. Two of the most common licensed programs are SEQUEST<sup>26</sup> and Mascot.<sup>27</sup> Both algorithms use the fragmentation patterns of MS/MS analysis to compare to the predicted fragmentation patterns of database protein sequences. A third algorithm that functions in a similar manner and is becoming popular is the open-source XTandem program.<sup>28</sup>

After the identified peptides are matched to protein sequences, there may be a need to further analyze the protein sequence to elucidate function, modification, or cellular location. One of the tools available for this is the basic local assignment search tool (BLAST).<sup>29</sup> BLAST can search both nucleotide and protein databases to identify protein homology, which is useful when the database used for peptide identification is insufficiently annotated. Additional information, such as subcellular localization can be found using the TargetP<sup>30</sup> localization predictor. Another useful program is the Gene Ontology Tool<sup>31</sup> to infer functional classification of proteins. More helpful

sequence analysis programs can be found at the Expert Protein Analysis System (ExPASy) proteomics server (<http://www.expasy.ch>). Additional tools for analysis of MS data can be found at the Protein Prospector (<http://prospector.ucsf.edu>), as well as helpful proteomics software at the Proteome Commons (<http://www.proteomecommons.org>).

## PROTEIN QUANTIFICATION

The area of differential proteomics has seen several advances in recent years. Three of the newer techniques can be used for differential quantitative analysis using MS (a technology that does not lend itself well to quantitative measurement unless internal standards are used). Each of the techniques involves differential labeling of proteins from different samples using stable isotopes, such as deuterium,  $^{15}\text{N}$ , or  $^{13}\text{C}$ . There are two ways to label proteins with these isotopes: biological incorporation, where cells are grown on media enriched with the isotope being used. The second method is known as chemical incorporation, where the isotopically labeled tag is added to proteins after extraction. Regardless of label incorporation method, corresponding labeled and unlabeled peptides will be detected in the mass spectrometer at the same time. Quantitative data are derived by comparing the ratio of areas of the MS peaks for labeled and unlabeled peptides.<sup>32</sup>

The first technique is stable isotope labeling by amino acids in cell culture (SILAC),<sup>33</sup> which is a biological incorporation method. Next is the isotope coded affinity tag (ICAT),<sup>34</sup> which involves a chemical incorporation of a deuterium-labeled (and unlabeled) reagent to cysteine residues. Last is a derivative of the ICAT method known as isotope tagging for relative and absolute protein quantitation (iTRAQ).<sup>35</sup>

The primary advantage of ICAT and iTRAQ labeling is the ability to label proteins that cannot be labeled using biological incorporation, such as human serum samples. ICAT also allows for decreasing the complexity of a complex biological mixture by the use of biotin affinity separation. The main disadvantage of ICAT compared to other labeling methods is the fact that only cysteine-containing peptides can be identified. The iTRAQ has the advantage over ICAT in being able to label all peptides in a mixture. It also allows for the analysis of four different samples at once, rather than just two with ICAT. The biggest disadvantage of iTRAQ is the more complex MS identification, as well as its increased cost.

Another widely used quantification technique is the two-dimensional difference gel electrophoresis (DIGE).<sup>36</sup> DIGE uses a system of fluorescent markers that bind to proteins in a sample, allowing for quantification of labeled proteins in a 2D gel upon excitation of the marker by a laser. Samples of interest can then be in-gel digested with a protease and analyzed by MS/MS.

## APPLICATION OF PROTEOMIC ANALYSIS TO FUNGAL SYSTEMS

There are several examples of proteomic analyses of fungal organisms to be found in the current literature. Many of these, such as an analysis of the obligate plant pathogen *Uromyces appendiculatus*, use some of the techniques described above.<sup>37</sup> In this analysis, proteins were extracted from uredospores, digested, and then separated by MudPIT. The analysis identified over 400 proteins, many of which are associated with protein production, such as translation factors, ribosomal proteins, and amino acid synthetases. These results led the authors to hypothesize that the uredospores exist in a suspended state of translation that allows the spore to begin protein production rapidly upon germination.

An analysis of the human pathogen *Candida albicans* incorporated 2DE separation of cell wall proteins prior to MS analysis.<sup>38</sup> In this study, the cell walls of the yeast and hyphae morphologies were subjected to protein extraction by SDS and DTT or cyanogen bromide (CNBr)/trypsin digestion. This study produced a total of 82 SDS/DTT-extractable cell wall proteins from both yeast and hyphal samples. Seven of these proteins were shown to be up-regulated in the yeast–hyphae transition, and two were downregulated. There were an additional 29 proteins identified from the CNBr/trypsin digestion of both cell types, 12 of which are hyphae-specific, and 6 are yeast-specific. These protein identifications have not only increased the understanding *C. albicans* biology, but also identified a heat-shock protein that is upregulated in the yeast–hyphae transition, but not at the mRNA level. These results suggest that this protein is regulated at a posttranslational level in the fungal cell wall.

Another analysis also focused on *C. albicans*, illustrating the applicability of proteomics to vaccine development.<sup>39</sup> In this study, an extract of yeast cell wall proteins was shown to be effective in protecting mice from infection. This study identified and characterized 20 proteins that reacted with antibodies from the serum of immunized animals. Many of the identified proteins were determined to play important roles in adhesion, cell-surface hydrophobicity, and immunogenic activity. These protein identifications have produced target antigens to be used in the development of a subcellular vaccine against *C. albicans* infection.

There are other examples of fungal proteomics, such as the analysis of proteins secreted by the phytopathogen *Sclerotinia sclerotiorum*.<sup>40</sup> In this study, both mycelial and secreted proteins were separated by 2DE. This analysis identified 18 secreted proteins, along with 95 mycelial proteins that provide insight into the fungal life cycle and pathogenicity. One protein had not been previously identified in the analysis of mRNA levels, highlighting the value of direct protein identifications, rather than protein presence inferred from transcript analysis.

The quantitative technique SILAC was used in a study of the complete proteome of *Saccharomyces cerevisiae*.<sup>41</sup> In this analysis, yeast cells were grown in normal media or media containing labeled lysine. The proteins collected from the cells were digested and the resulting peptides were analyzed on a linear ion trap-FT mass spectrometer capable of extremely high peptide mass accuracy. Peptides were identified by MS/MS fragmentation, resulting in identification of over 2,000 *S. cerevisiae* cytoplasmic proteins. These identifications included low-abundance proteins corresponding to about 100 protein copies per cell.

Another recent analysis used SILAC-like stable isotope labeling for protein quantification in *Schizosaccharomyces pombe*.<sup>42</sup> In this study, fungal cells were treated with Cd<sup>2+</sup> and labeled with deuterated leucine to determine what effect the toxic metal had on protein production. This study identified 106 proteins that were upregulated and 55 that were downregulated in response to Cd<sup>2+</sup> treatment. In addition, 28 of the upregulated proteins were revealed to be proteins involved in detoxification of reactive oxygen species (ROS) or repair of damaged cellular components. This study serves to highlight the applicability of proteomics to analysis of environmental effects on cellular metabolism.

There are multiple examples of proteins of interest identified from various cellular preparations of *Coccidioides*. Included among these is the coccidioides-specific antigen (CSA), which was first isolated from the soluble wall fraction of infectious arthroconidia<sup>43</sup> in 1989 by acetone extraction followed by electrophoresis separation and protein removal by electroelution. It was not until 1995 that the protein sequence for CSA was determined by sequencing of the N-terminal and proteolytically produced peptides, as well as sequence determination of isolated cDNA.<sup>44</sup> Another protein, Antigen 2 (Ag2), was first identified in 1978 by two-dimensional immunoelectrophoresis (IEP) from the crude antigen preparations coccidioidin and spherulin,<sup>45</sup> and also found in the alkali-soluble, water-soluble mycelial and spherule extracts.<sup>46</sup> Ag 2 was not sequenced until 1996.<sup>47</sup> A parallel antigen discovery of the proline-rich antigen (PRA) was identified from a toluene spherule lysate.<sup>48</sup> PRA was also sequenced in 1996,<sup>49</sup> at which point it was discovered that Ag2 and PRA were the same protein. These studies highlight some of the important protein discoveries made in *Coccidioides*, and provide much of the groundwork for future protein antigen identifications.

While there are numerous examples of protein antigen identifications in *Coccidioides*, more modern proteomic analyses using MS have only recently been reported. These studies have primarily focused on identification of antigenic proteins. The analysis of T-cell reactive antigens associated with the spherule cell wall by 1D and 2D electrophoresis protein separations followed by peptide identification via MS/MS identified a protective aspartyl protease (Pep1).<sup>50</sup> Another analysis of seroreactive spherule cell wall proteins separated by 2DE and analyzed by MS/MS identified two more protective protein antigens, phospholipase B (Plb) and alpha-mannosidase (Amn1), in addition to Pep1, all

of which were shown to be protective in mice as a multivalent recombinant protein vaccine.<sup>51</sup> A 2D DIGE analysis of differential protein expression between the mycelial and spherule phases of *C. posadasii*, resulted in the identification of a new vaccine candidate protein, a peroxisomal matrix protein known as Pmp1, also shown to be protective in mice against coccidioidal infection.<sup>52</sup> Immunoblot analysis of a 2D gel of the thimerisol-inactivated spherule vaccine (T27K) was analyzed by MS, resulting in the identification of a putative Cu, Zn superoxide dismutase (SOD)<sup>53</sup> as well as Amn1.<sup>54</sup> A summary of the identifications of Amn1 and SOD is included elsewhere in the proceedings. Another study<sup>55</sup> purified N-glycan containing glycoproteins from T27K by lectin-affinity chromatography followed by SDS-PAGE separation. From this study, a 60-kDa protein component was identified by MS, with homology to a 1,3 glucanosyltransferase from *C. posadasii* and other fungi.

## FUTURE DIRECTIONS

The above list of fungal proteomic analyses, while not exhaustive, provides a glimpse into the wide range of experimental questions that can be answered by deliberate application of proteomic techniques. Future proteomic analyses of *Coccidioides* and other fungal species will undoubtedly prove beneficial to vaccine development efforts, as well as increase the understanding of the biology of these organisms. Current efforts are under way at the University of Arizona to analyze the spherule cell wall proteome, and to comprehensively analyze differential protein expression between the mycelial and spherule phases of *Coccidioides posadasii* by stable isotope labeling. These types of studies will likely prove beneficial to *Coccidioides* vaccine development, as to well as to other fungal systems.

Future proteomic studies of the *Coccidioides* species are likely to focus on specific proteins involved in virulence that could lead to development of novel drug treatments. Many of the techniques presented here would also be effective in elucidating protein biomarkers for coccidioidal infection or perhaps provide clues to the nature of sexual reproduction in these fungal species. Regardless of the scope of studies yet to come, modern proteomic techniques are likely to be included in the tools researchers use to answer important questions about this important fungal pathogen.

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