

5

Fungal Proteomics

5.1 Introduction

The term “proteomics” refers to the large-scale study of the proteins present in an organism. Consequently, “fungal proteomics” can be defined as the study of the intracellular and extracellular protein complement of fungi. Historically, individual proteins were isolated by a combination of chromatographic techniques, enzymatically characterized, and subjected to N-terminal or partial amino acid sequence analysis for identification.

However, the advent of genome sequencing and protein mass spectrometry (MS), allied to high-resolution separation techniques for proteins (e.g. molecular mass-based separation by electrophoresis) has meant that thousands of proteins can be simultaneously isolated, separated, and identified from an individual fungus. Although this large-scale approach has drawbacks, such as the generation of huge amounts of data, it represents the current situation with respect to the study of fungal proteomics.

The strategies for undertaking fungal proteomic investigations are continually evolving, but all share the following themes:

- Availability of a full or partial genome sequence (or cDNA) for the fungus of interest, or establishment of the extent of fungal genomic information on related fungi in publicly available databases (e.g. PubMed; <http://www.ncbi.nlm.nih.gov/pubmed>).
- Optimization of protocols for both fungal culture and intracellular and/or extracellular protein isolation.
- Separation of proteins by techniques such as chromatography or electrophoresis (e.g. sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), Figure 5.1). This step is optional in the case of shotgun proteomics.
- Fragmentation of individual proteins into peptides by digestion with the proteolytic enzyme trypsin.
- Analysis of each peptide mixture by liquid chromatography–tandem mass spectrometry (LC-MS/MS). This technique separates the peptides from one another, determines the mass, and sequence, of each, and then compares these data to DNA or cDNA databases.

5.2 Protein Isolation and Purification

5.2.1 Cell Lysis Strategies

The extraction or isolation of proteins from fungi is a prerequisite for subsequent analysis. Unlike animal cells, which do not possess a cell wall, and where the cell membrane can be readily lysed using detergents, the rigid cell wall of fungi¹²² Fungal PrOTeOMiCS represents a significant barrier to the efficient extraction of intracellular proteins.

Consequently, a number of vigorous techniques have been devised to enable the release of proteins from fungal mycelia, including hyphal maceration in liquid N₂ using mortar and pestle, bead-beating, sonication, and rapid pressure changes (**French Press technology**). One or a combination of these physical techniques must be used to disrupt fungal mycelia, which, allied to strict temperature control (2–8 °C), use of protease inhibitors, pH control, and optimal mycelia:extraction buffer ratio, will yield efficient release of intracellular contents.

Mycelial lysis is generally followed by high-speed centrifugation to remove insoluble material such as intact mycelia, cell wall fragments, or cell debris, to yield a clarified, protein-rich supernatant. This supernatant can then be further processed by ammonium sulfate fractionation, dialysis, or volume reduction by ultrafiltration, prior to protein purification by chromatographic techniques.

5.2.2 Chromatography

A detailed description of chromatographic techniques is beyond the scope of this chapter; however, ion-exchange chromatography (cation or anion exchange) facilitates protein separation by charge, gel filtration enables protein separation based on molecular mass, and affinity chromatography allows protein isolation by virtue of the specific affinity between the protein of interest and an immobilized ligand. Table 5.1 provides information of the relative merits and demerits of each chromatographic approach. Once the protein of interest has been isolated, it can then be characterized in terms of activity (if an enzyme), sequence, immunologically, or by protein–protein interaction. Chromatography is generally used for the preparative isolation of proteins for further use, but more recently these techniques have been coupled to MS-based proteomics for on-line or off-line separation of peptides prior to **LC-MS/MS**.

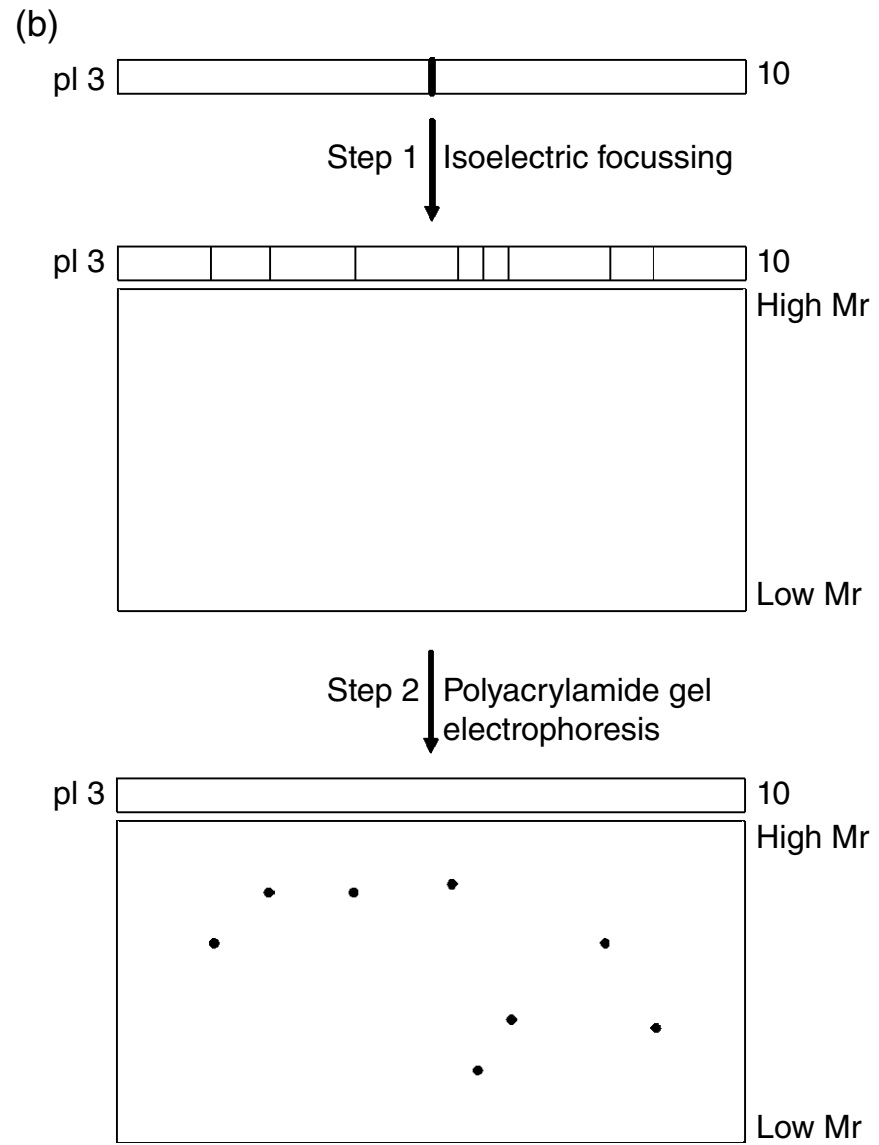
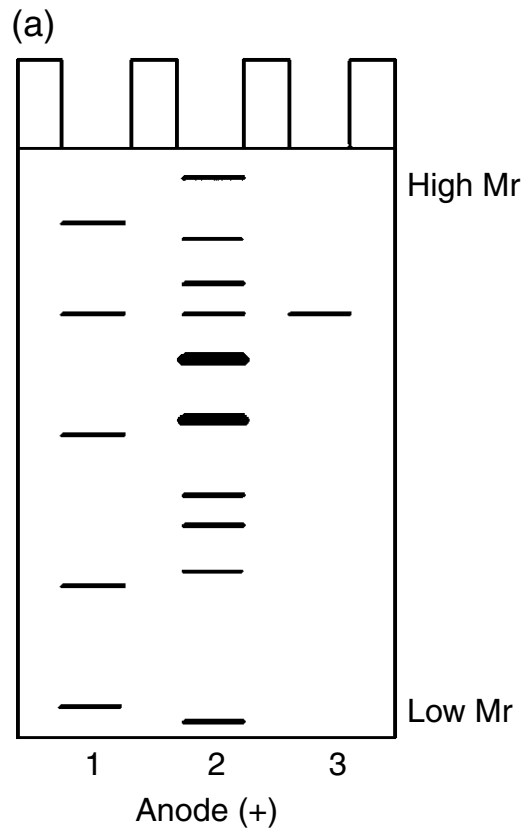
Table 5.1 Advantages and disadvantages of alternative chromatographic techniques for fungal protein isolation.

Chromatographic technique	Advantages	Disadvantages
Ion exchange	High resolution, high speed, high protein capacity resins, crude protein preparations suitable for use	Salt interference, total protein purity rarely achieved
Gel filtration	Separation based on molecular mass, high purity achievable, no salt interference	Low resolution, pre-fractionation required, time-consuming
Affinity	Excellent protein purity achievable, rapid, no pre-fractionation required	Requires affinity ligand, harsh protein release conditions
Immunoaffinity	Excellent protein purity achievable, rapid ¹	Requires protein-specific purified IgG, immunoaffinity-purified protein must be IgG-free

¹Chromatography is rapid; however, antibody generation may be time-consuming.

5.2.3 Protein Extraction Prior to 2-D Polyacrylamide Gel Electrophoresis (2D-PAGE)

Protein separation by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is explained in detail in Section 5.3. This important separation technique exploits both protein charge and molecular mass to yield individually resolved proteins, in sufficient amount, which can be analyzed and identified by MS following enzymatic digestion by trypsin. This represents a significant advantage over chromatographic purification which can take days or weeks to complete and often yields only one or a few pure proteins. Fortunately, mycelial protein can be extracted directly into reagents which are compatible with subsequent separation by 2D-PAGE. Here, mycelia are ground in liquid N₂ and sonicated in “solubilization buffers” which contain high concentrations of urea, thiourea, and detergents, along with ampholytes. Protein extracts prepared in this way can be applied directly to isoelectric focussing strips (pH range 4–7 or 3–10) to facilitate separation by isoelectric point prior to subsequent separation by molecular mass via protein electrophoresis (Figure 5.1). to lower the concentration of the denaturing agent (e.g. to 1 M urea), followed by incubation with the protease trypsin. This enzymatically fragments all protein present into constituent peptides by cleaving the peptide bond on the carboxyl side of lysine or arginine residues. This peptide mixture can then be subjected to LC-MS/MS analysis to facilitate global protein identification. Sample clean-up is often carried out using C18 resins (e.g. C18 ZipTips) to remove residual salts ahead of analysis.



5.2.5 Subcellular Fractionation

Separating mycelial lysates into distinct fractions can serve a dual purpose in proteomic analyses. This fractionation tool can allow targeted investigations of specific organelles (e.g. mitochondria) or subcellular domains (e.g. microsomes) within the organism. In addition, this technique serves to reduce sample complexity, which in turn enables wider proteome coverage to be achieved. One method employed for subcellular fractionation is **differential centrifugation**, a tool that sequentially separates components in order of decreasing density.

A sample protocol for recovery of the microsomal fraction from the filamentous fungus *Aspergillus fumigatus* is shown below. Total cell lysates are generated initially, followed by a medium-speed centrifugation step to pellet dense material (cell wall, nuclei, etc.), and finally high-speed centrifugation to pellet membrane-associated material (microsomal fraction). Using this protocol, 710 unique proteins were detected from microsomal fractions of *A. fumigatus* using the Orbitrap LTQ XL mass spectrometer, and this number has the potential to be increased using the higher resolution mass spectrometers such as the ThermoQ-Exactive instrument.

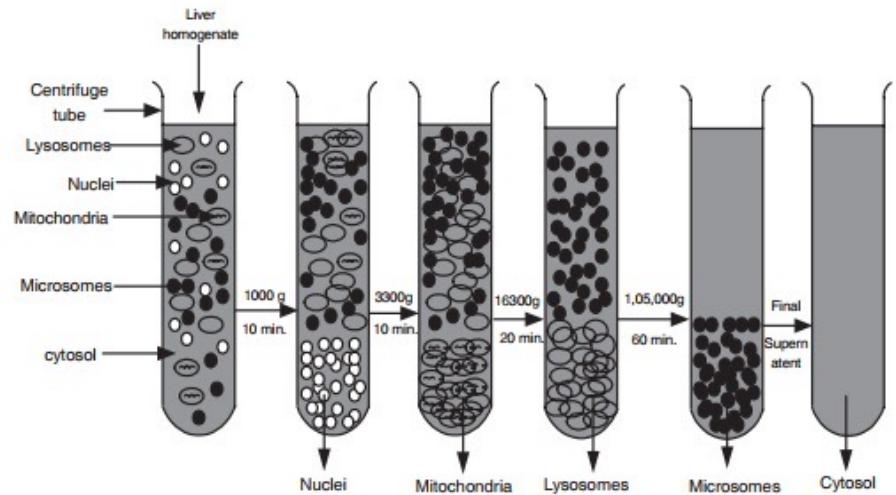


Fig. Differential sedimentation of subcellular organelles

5.2.6 Protein Recovery from Culture Supernatants

In addition to the production and presence of mycelial (intracellular) proteins, fungi secrete a wide range of enzymes into the extracellular environment; indeed the pattern of secreted enzymes is often dependent on the available carbon source. Fungal culture can take place on either solid matrices (solid-state fermentation (SSF)) or in liquid culture, sometimes referred to as submerged fermentation. In both scenarios, the concentration of secreted enzymes is generally low (nanograms to micrograms per milliliter) and so a concentration step is often required prior to subsequent analysis and characterization. Initial enzyme recovery from SSF is generally via resuspension in aqueous buffered solutions (occasionally containing low concentrations of detergents). Once resuspended SSF material or submerged culture supernatants are available, enzyme concentration is effected by

- (1) ammonium sulfate precipitation,
- (2) protein ultrafiltration
- (3) Lyophilization
- (4) TCA precipitation.

Once one or more of the concentration steps has been performed, and the volume has been reduced by up to a factor of 50–100, analytical tests such as protein estimation or protein electrophoresis are performed to estimate and visualize all proteins present. It should be noted that many fungi, especially basidiomycete species, produce large amounts of extracellular carbohydrate polymers which can interfere with the isolation of secreted enzymes. Consequently, a high-speed centrifugation step is often used to remove this material prior to protein concentration when investigating the extracellular proteome (secretome) of many fungal species.

5.3 Electrophoretic Techniques

5.3.1 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

5.3.2 2D-PAGE

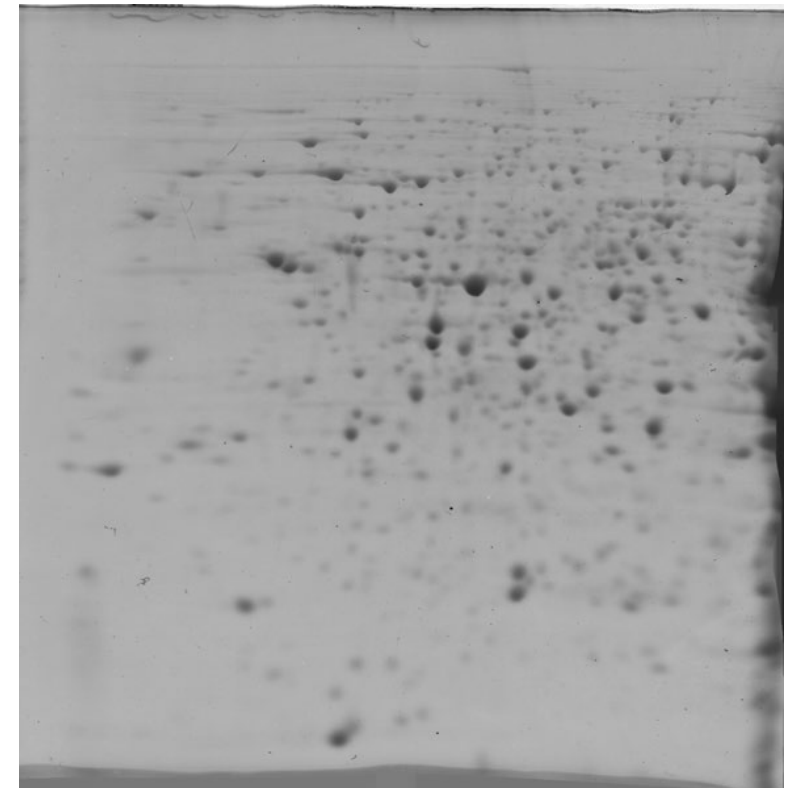
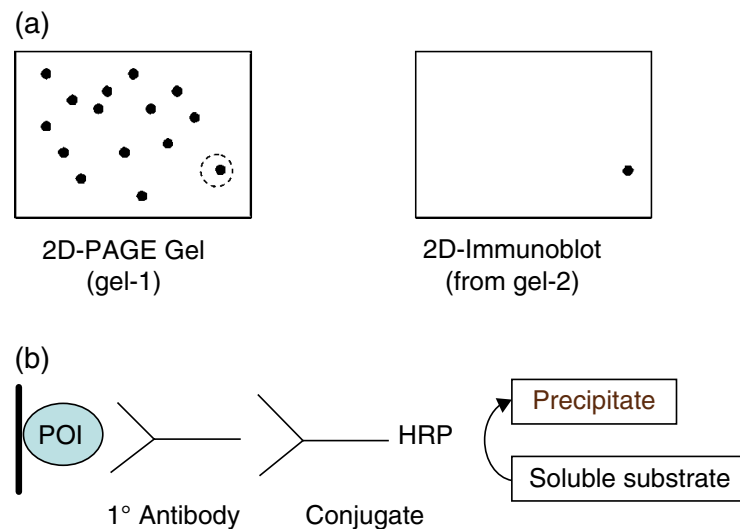


Figure 5.2 Immunoproteomics. (a) Duplicate 2D-PAGE analysis is performed and gel-1 is stained with Coomassie brilliant blue dye, while gel-2 is subjected to electrotransfer onto a suitable membrane. This membrane is subsequently probed with antisera using an immunoblot procedure as shown in (b) to detect immunoreactive proteins (2D-immunoblot). The corresponding protein (circled) in gel-1 can then be excised and identified by protein MS.

5.4 Protein Mass Spectrometry

5.4.1 Genome Databases

Table 5.2 Genomic, proteomic, and general fungal websites.

Database	URL address
<i>Aspergillus</i> genomes (CADRE)	www.cadre-genomes.org.uk
<i>Aspergillus</i> genomes (AspGD)	www.aspergillusgenome.org
Multifungal genome database	http://mips.gsf.de/genre/proj/fungi/fungal_overview.html
<i>Aspergillus nidulans</i>	www.broadinstitute.org/fungal-genome-initiative/aspergillus-genome-projects
<i>Aspergillus niger</i>	http://genome.jgi-psf.org/Aspni5/Aspni5.home.html
US Government Genome Sequencing Initiative	www.nhlbi.nih.gov/research/resources/nhlbi-precision-medicine-initiative/topmed/wgs
Fungal genomes	http://fungalignomes.org/blog/
FungalGenetics Stock Centre	www.fgsc.net
UniProt	www.uniprot.org
ExPASy	http://expasy.org/
Mascot	www.matrixscience.com
MycCosm at JGI	http://genome.jgi.doe.gov/programs/fungi/index.jsf

5.4.2 Protein Digestion

- (1) inhibition of peptide bond cleavage
- (2) loss of large hydrophobic peptides by adherence to plastic or insolubility
- (3) release of multiple short peptides of low molecular mass (<300 Da)
- (4) nonspecific binding of peptides to filters used for sample preparation prior to MS analysis, and
- (5) modified K or R residues.

From an instrumentation viewpoint, peptides may not efficiently ionize during MALDI MS analysis or can be irreversibly bound to LC columns during LC-MS, thereby resulting in absence of detection.

5.4 Protein Mass Spectrometry

5.4.3 Mass Spectrometry

5.4.4 MALDI-ToF Mass Spectrometry

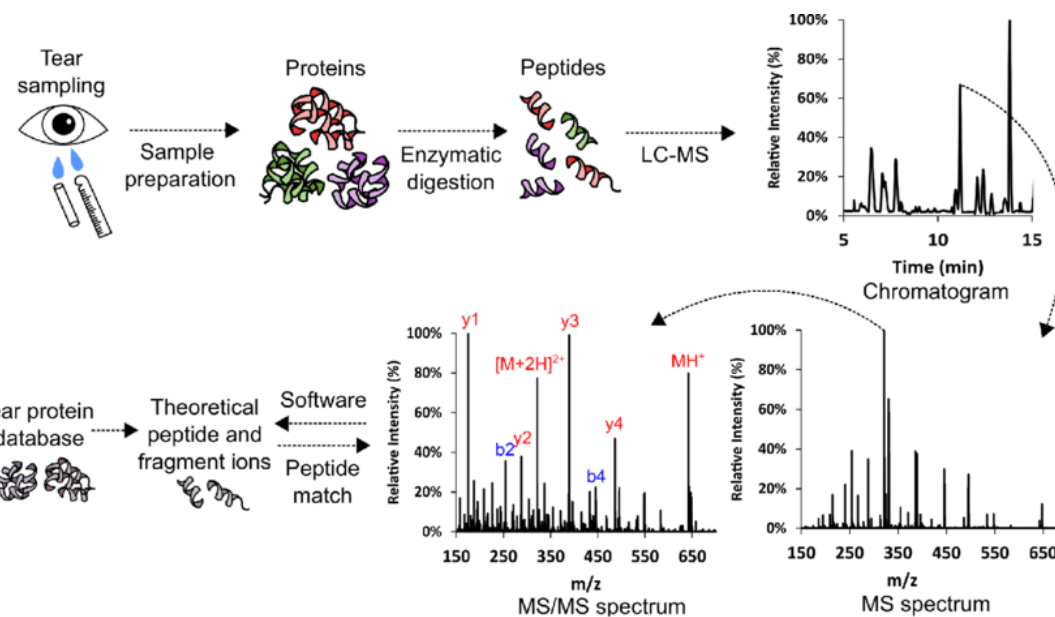
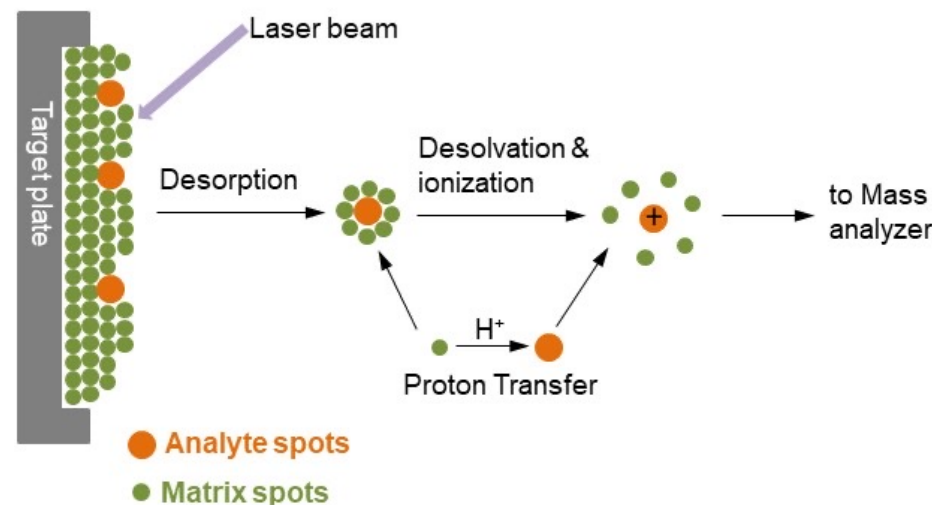
matrix-assisted laser desorption ionization–
time-of-flight mass spectrometry

<https://tsen.in.th/เทคนิคมาลดี-ทอป-เอ็มเอส-maldi-t/>

5.4.5 Electrospray Mass Spectrometry

[https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_\(Analytical_Chemistry\)/Instrumentation_and_Analysis/Mass Spectrometry/Mass Spectrometers \(Instrumentation\)/Electrospray Ionization Mass Spectrometry](https://chem.libretexts.org/Bookshelves/Analytical_Chemistry/Supplemental_Modules_(Analytical_Chemistry)/Instrumentation_and_Analysis/Mass_Spectrometry/Mass_Spectrometers_(Instrumentation)/Electrospray_Ionization_Mass_Spectrometry)

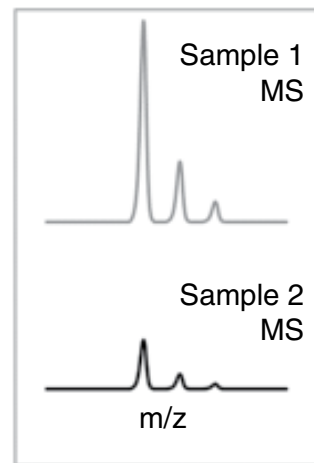
5.4.6 Shotgun Proteomics



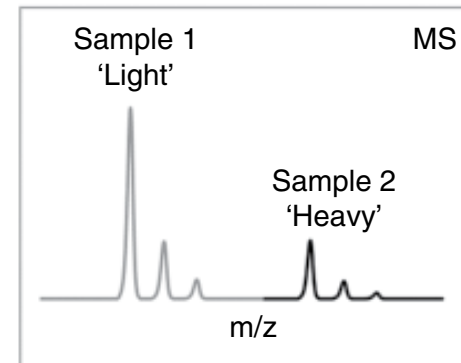
5.4.7 Quantitative Proteomics

- (1) to investigate the effect of culture conditions on the extent of protein expression
- (2) to study altered global or specific protein expression following gene deletion,
- (3) of altered virulence factor expression during infection.

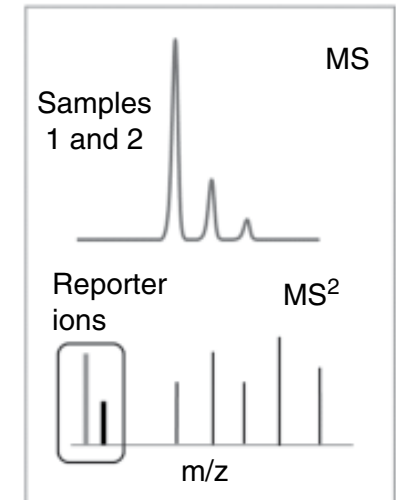
5.4.8 Label-Free Proteomics



Label-free



Isotopic labels
(SILAC, ICAT)



Isobaric labels
(iTRAQ, TMT)

5.5 Fungal Proteomics