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Isolation and Enrichment of Secreted Proteins from Filamentous Fungi

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Summary

Filamentous fungi have been recognized as extraordinary producers of secreted proteins and are known to produce novel proteins and enzymes through dispensable metabolic pathways. Here, methods are described for the isolation and enrichment of samples of secreted proteins from cultures of filamentous fungi for analysis by gel electrophoresis and mass spectrometry techniques. These methods can be readily applied to the study of differential protein expression and secretion and metabolic pathways in filamentous fungi by proteomic approaches.

Key Words: Exoproteome; extracellular proteins; protein deglycosylation; protein precipitation; secreted proteins; secretome; gel electrophoresis.

1. Introduction

Protein secretion plays an important role in filamentous fungi, particularly in nutrition, as secreted enzymes degrade complex biological molecules to serve as carbon and nitrogen sources. Filamentous fungi are known for their ability to secrete a broad spectrum of enzymes, the majority of which are hydrolytic, into the extracellular matrix ***(1)***. This ability has been widely exploited by the biotechnology industry for the production of enzymes for commercial and industrial use. Most commonly, filamentous fungi secrete proteins via a classical secretory pathway ***(2)*** and most, if not all, of the secreted proteins are glycosylated, containing modifications such as oligomannose *N*- and *O*-glycans ***(3)***. These attached sugars increase the stability of the secreted proteins

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and provide resistance to environmental influences, as well as increase their solubility in the culture media. Although many studies of protein secretion have been carried out in yeast and animal systems, studies on protein secretion by filamentous fungi are limited ***(4)***. Typical studies have focused on the identification, purification and characterization of secreted proteins, but only a few studies have been conducted on the global analysis of fungal extracellular proteomes. Proteomic studies on the analysis of secreted proteins from a number of fungi, including *Aspergillus flavus* ***(5,6)***, *A. oryzae* ***(7)***, *Fusarium graminearum* ***(8),*** and *Pleurotus sapidus* ***(9)***, have recently appeared in the literature. Much of the delay in the use of proteomic techniques for the study of fungal protein expression in general can be attributed to the fact that there is a lack of complete and publicly available genome sequences from filamentous fungi. It is expected that as the number of published fungal genomes increases, proteomic studies on both intracellular and extracellular proteins will follow.

Within their high capacity to produce secreted proteins, filamentous fungi are able to express and secrete proteins for "dispensable" metabolic functions ***(10)***. These enzymes participate in pathways that are either not required for growth or are only required for growth under a limited range of conditions. These changes in protein secretion provide an excellent platform for the systematic study of the overall protein secretion expression (secretome or exoproteome) as a function of culture conditions. Proteomic analysis using two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) has proven to be the most powerful and sensitive method for the identification of proteins in complex mixtures. 2-DE provides an excellent platform to assess differential secreted protein expression. Although the number of proteins that can be analyzed by 2-DE is still limited to 1,000–2,000 on one gel, the maximum number of secreted proteins by a filamentous fungi under a given set of conditions is well within this range, as a recent analysis of the genome of *Aspergillus niger* identified only about 400 putative secreted proteins from a total of about 5,100 genes ***(11)***. Obtaining MALDI-TOF MS data from tryptic digests of gel bands and spots and searching against online protein databases can identify 2-DE separated proteins. The success of peptide mass fingerprinting depends on the detection of a representative set of peptide masses derived from a protein and that the protein in question is known (it exists in a protein database). Alternatively, proteins can be identified by MS/MS with custom databases that contain sequences of all publicly known fungal proteins or by *de-novo* sequencing using this technique.

Several technical issues need to be considered when studying the secretome of filamentous fungi by proteomic analysis. First, although fungi have the ability to secrete large amounts of protein, these proteins are highly diluted in the culture medium. Second, glycosylation poses a problem for visualization of proteins separated by gel electrophoresis, as glycosylated samples tend to

smear in gels. In addition, glycosylation can protect proteins against proteolysis, making the identification of these proteins more difficult as peptide mass fingerprinting relies on efficient protease digestion before mass spectrometry ***(12)***. Also, the attached sugars greatly increase the size of generated peptide fragments, adding another layer of complexity in their identification by mass spectrometry. These problems can be overcome by concentrating the media, most commonly by lyophilization or ultrafiltration, and by removing the sugars with commercially available glycosidases or by chemical deglycosylation methods.

Here we describe a general procedure for the isolation and enrichment of secreted proteins from cultures of filamentous fungi for proteomic analysis by gel electrophoresis and mass spectrometry, including protocols for protein deglycosylation. The methods described here can be easily adopted for the isolation and characterization of the secretome of filamentous fungi grown under varied conditions.

2. Materials

2.1. Filtration and Lyophilization

1. Miracloth (Calbiochem, EMD Biosciences, Inc., San Diego, CA) or No. 2 Whatman filter paper.
2. Lyophilizer

2.2. Centrifugation and Ultrafiltration

1. Centricon Plus-70 Centrifugal Filter Device (Millipore, Billerica, MA).
2. Centrifuge

2.3. Precipitation with Trichloroacetic Acid

1. 20% (w/v) Trichloroacetic acid (TCA) (Sigma-Aldrich, St. Louis, MO), stored at 4°C.
2. 70% Ethanol, stored at –20°C.
3. Acetone

2.4. Precipitation with Methanol and Chloroform

1. Methanol
2. Chloroform

2.5. Enzymatic Deglycosylation

1. Ultrafree-0.5 centrifugal filter devices with Biomax-5 membranes 5,000 NMWL (Millipore, Billerica, MA).

2. Peptide: *N*-glycosidase F (PNGase F) (New England BioLabs, Inc., Ipswich, MA). PNGase F is provided with 10× glycoprotein denaturing buffer (0.5% SDS, 1% β-mercaptoethanol), 10× G7 reaction buffer (50 m*M* sodium phosphate, pH 7.5), 10% Nonidet P-40 (NP-40), and a solution of PNGase F *(see* **Note 1**).

2.6. Chemical Deglycosylation *(see* Note 2*)*

1. Reacti-Vial Reaction Vials (5 mL) (Pierce Chemical Co., Rockford, IL).
2. Trifluoromethanesulfonic acid (TFMS) (Sigma/Aldrich, St. Louis, MO, USA) *(see* **Note 3**).
3. Anisole, anhydrous (Sigma/Aldrich, St. Louis, MO), stored at 4°C.
4. 60% (v/v) Pyridine solution, stored at –20°C.
5. Diethyl ether, stored at –20°C.
6. 95% Ethanol, stored at 4°C.

2.7. SDS-PAGE

1. SDS Sample Reducing Buffer: Mix 4.05 mL of deionized water, 1.25 mL 0.5*M* Tris-HCl, pH 6.8, 2.50 mL glycerol, 2.00 mL 10% SDS and 0.20 mL 0.5% (w/v) bromophenol blue.
2. β-Mercaptoethanol

2.8. Two-Dimensional Gel Electrophoresis

1. 2-DE Buffer: 8*M* urea, 2% (w/v) CHAPS, 50 m*M* dithiothreitol, 0.2% (w/v) 100× Bio-Lyte 3–10 ampholytes (Bio-Rad Laboratories, Inc., Hercules, CA), 0.001% (w/v) bromophenol blue.

3. Methods

This chapter describes general-purpose sample preparation methods for the isolation and enrichment of secreted proteins from cultures of filamentous fungi. The methods described in the following section can be divided into (1) isolation and concentration by filtration and lyophilization or ultrafiltration, (2) precipitation, and (3) deglycosylation of concentrated protein samples by enzymatic (PNGase F) or chemical deglycosylation using TFMS acid ***(13)***. The samples can then be further analyzed by gel electrophoresis and mass spectrometry by established techniques described in other chapters. A schematic representation of these protocols is shown in **Fig. 1**.

3.1. Isolation and Concentration of Supernatant Broth

1. After the studied filamentous fungus has been grown in liquid culture media for the desired time, the broth containing the secreted proteins is collected by filtration

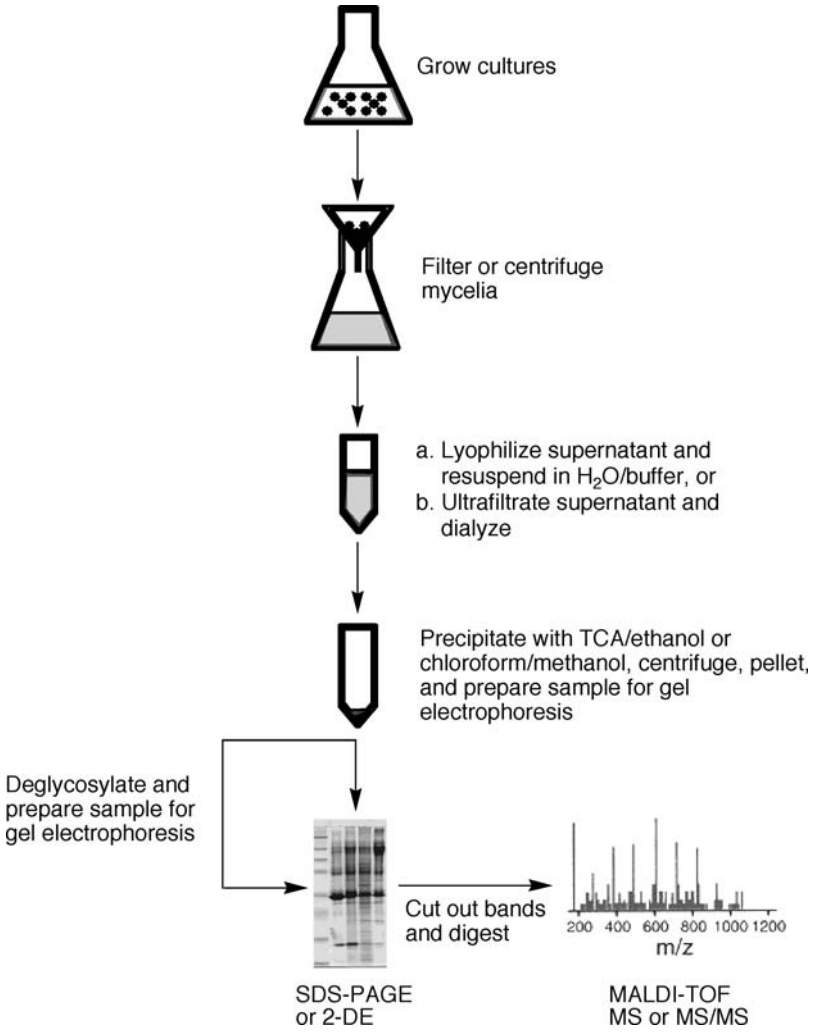

Fig. 1. Simplified schematic for the isolation and enrichment of secreted proteins from cultures of filamentous fungi for proteomic analysis (*see* **Methods** section for details).

through a Miracloth or No. 2 Whatman filter paper. Alternatively, the fungal mycelia can be separated from the supernatants by centrifugation at 10,000*g* for 10 min. (*see* **Note 4**).

2. The supernatants are concentrated by lyophilization (steps 3–5) or ultrafiltration (steps 6–8).
3. For lyophilization, place the filtered supernatants in individual round bottom flasks and freeze in liquid nitrogen. (*see* **Note 5**).

4. Place the round bottom flasks on the lyophilizer until they are completely dry.
5. Redissolve the contents of the round bottom flasks in a minimal amount of deionized water, and store at –20°C until further analysis.
6. For ultrafiltration, place the filtered supernatant in a Centricon Plus-70 centrifugal filter device to a maximum of 70 mL.
7. Centrifuge at 3,500*g* until the desired final volume is achieved.
8. The concentrated supernatant can be washed with an appropriate buffer (e.g., 50 m*M* phosphate buffer, pH 6.0) and reconcentrated to remove excess salts, pigments, and metabolites. (*see* **Note 6**).

3.2. Protein Precipitation

Protein precipitation is an efficient method for the removal of most contaminants, including detergents, salts, peptides, lipids, and phenolic compounds from protein samples. Either one of the methods described below can be used to precipitate the secreted proteins from the concentrated broth supernatant in preparation for gel electrophoresis analysis. It should be noted that no method will precipitate all proteins and some proteins will be difficult to resuspend following precipitation.

*3.2.1. Precipitation with Trichloroacetic Acid (*see **Note 7***)*

1. Pipet 0.3 mL sample of concentrated broth supernatant into a 1.5-mL siliconized microcentrifuge tube and add an equal amount of cold TCA solution.
2. The mixture is incubated for 2 h at –20°C to allow the proteins to precipitate. After 2 h, allow samples to thaw if lightly frozen.
3. Centrifuge for 10 min at 14,000*g*.
4. The samples are decanted, and 1 mL of cold 70% ethanol is added, vortexed, and recentrifuged for 3 min.
5. Step 4 is repeated 3 times.
6. To completely dry the secreted protein pellet, add 1 mL of acetone, vortex and centrifuge for 1 min. The acetone is decanted, and the pellet is allowed to air dry for 30 min.
7. The dried pellet can be stored at –20°C until further analysis.
8. For electrophoresis analysis, the pellet is dissolved in SDS-PAGE or 2-DE sample buffer, as described below (*see* **Subheading 3.5** and **Note 8**).

3.2.2. Precipitation with Chloroform/Methanol

1. To 100 µL of the concentrated supernatant in a siliconized microcentrifuge tube, add 400 µL of methanol, 100 µL of chloroform, and 300 µL of H_2O, and mix well.
2. Incubate at 4°C for 5 min and centrifuge at 9,000*g* at 4°C for 2 min.

3. The upper phase is carefully removed and discarded. Add another 300 µL of methanol to the rest of the lower chloroform phase and the interphase with the precipitated protein and mix well.
4. Incubate at 4°C for 5 min and pellet the proteins by centrifugation at 13,000*g* for 5 min at 4°C. The supernatant is removed and the protein pellet is dried under a stream of air.
5. The dried pellet can be stored at –20°C until further analysis.
6. For electrophoresis analysis, the pellet is dissolved in SDS-PAGE or 2-DE sample buffer (*see* **Subheading 3.5**).

3.3. Protein Deglycosylation

3.3.1. Enzymatic Protein Deglycosylation with PNGase F

1. Concentrate a 500 µL sample of the concentrated supernatant broth to 50 µL using a Ultrafree-0.5 with a Biomax-5 membrane (5,000 NMWL) centrifugal filter device by centrifugation at 12,000*g* at 4°C. To desalt the sample, add 400 µL of deionized water to the concentrated sample and centrifuge again until a final volume of 50 µL is reached.
2. The concentrated, desalted solution is removed from the centrifugal filter device and placed in a 600 µL siliconized microcentrifuge tube.
3. Enzymatic digestion is carried out as follows and according to the manufacturer's instructions (New England BioLabs, Inc.): 15 µL of denaturing buffer is added to the sample and boiled at 100°C for 10 min. To this sample, 3.5 µL of G7 buffer and 3.5 µL of NP-40 buffer are added, and the samples are digested with 1,000 U of PNGase F (*see* **Note 9**) for 18 hours at 37°C.
4. Following deglycosylation, the samples are precipitated as described above (see **Subheading 3.3**).

3.3.2. Chemical Protein Deglycosylation with Trifluoromethanesulfonic Acid

1. Glycoprotein samples should be relatively free of salts, minerals and detergents. Samples must also be completely dried. Lyophilize protein sample in a 5-mL Reacti-vial.
2. Incubate lyophilized sample with 0.3 mL of cold anisole and 0.6 mL of cold TFMS at 0°C in an ice-bath for 4 h under nitrogen with occasional shaking.
3. The reaction mixture is cooled to below –20°C by placing in a dry ice-ethanol bath and neutralized by slowly adding 1.2 mL of cold 60% aqueous pyridine solution.
4. The deglycosylated peptides are freed of reagents and low-molecular weight sugars by adding 2 mL of cold diethyl ether. The suspension is vortexed and extracted twice with cold ether.

5. The aqueous layer is lyophilized, redissolved in deionized water, and precipitated as described in **Subheading 3.3**.

3.4. Preparation of Samples for Gel Electrophoresis Analysis (see Note 10)

3.4.1. SDS-PAGE

1. Add 50 µL of β-mercaptoethanol to 950 µL SDS Reducing Buffer before use.
2. Dissolve the precipitated protein pellet in the appropriate volume of SDS reducing sample buffer determined by the size of the gel and the system used.
3. Boil sample for 4 min.

3.4.2. Two-dimensional Gel Electrophoresis

1. Dissolve the precipitated protein pellet in the appropriate volume of 2-DE Buffer determined by the size of the IPG strip and the system used for isoelectric focusing (*see* **Note 11**).
2. Incubate the sample for 2 h at room temperature and remove any insoluble material by centrifugation at 10,000*g* for 10 min.

4. Notes

1. PNGase F is an amidase that cleaves between the innermost *N*-acetylglucosamine and asparagine residues of high mannose, hybrid, and complex oligosaccharides from *N*-linked glycoproteins. PNGase F is the enzyme of choice for removing most *N*-linked oligosaccharides. Although secreted proteins could contain *O*-linked oligosaccharides, a recent study done on commercial cellulose enzyme preparation from the filamentous fungus *Trichoderma reesei* demonstrated that PNGase F treatment was superior to other enzymatic or chemical deglycosylation treatements in terms of yielding peptides through MALDI-MS that resulted in actual protein identification when searched against a database ***(12)***.
2. For chemical deglycosylation, the use of a commercially available kit is recommended. Two of such kits are the GlycoProfile IV, Chemical Deglycosylation Kit from Sigma/Aldrich (Sigma/Aldrich, St. Louis, MO, USA) and Glycofree Chemical Deglycosylation Kit from ProZyme, Inc. (San Leandro, CA, USA).
3. Trifluoromethanesulfonic acid is a strong acid, highly corrosive and hygroscopic. Protective goggles, laboratory coat and gloves should be worn when working with TFMS.
4. Typically, centrifuging is used to separate cells, in this case mycelia, from the supernatants. However, centrifuging does not work as well for filamentous fungi, as mycelia do not pack well and can float to the surface of the supernatant; therefore, filtering is preferred over centrifugation.

5. Culture supernatants in the round bottom flasks should be frozen by spinning the flask while immersing in liquid nitrogen to ensure even distribution of the supernatant in the flask. This will allow better lyophilization of the samples.
6. An alternate method for desalting is dialysis. Concentrated supernatant broth can be dialyzed against water or any appropriate buffer at 4°C.
7. An alternate protocol for precipitation of fungal secreted proteins by trichloroacetic acid has been described by Suárez et al. (2005) ***(14)***. In this protocol, the concentrated, dialyzed and lyophilized culture filtrate is resuspended in 20% TCA in acetone containing 0.2% dithiothreitol (DTT), stored at –20°C. The suspension is kept at –20°C overnight. The sample is centrifuged at 16,000*g* at 4 C for 10 min and the resulting pellet is washed three times with acetone containing 0.2% DTT. The supernatants are removed by centrifugation and the pellet is dried overnight at room temperature. The pellet is dissolved in SDS-PAGE or 2-DE sample buffer before gel electrophoresis analysis (*see* **Subheading 3.5**). In this protocol, acetone is used to increase the solubility of interfering organic compounds and increase protein precipitation and DTT is included to prevent protein modification.
8. TCA precipitation allows for further concentration of the proteins in the sample, as well as for removal of non-protein substances, salts and other agents that may interfere with electrophoresis separation. Care should be taken to make sure the protein pellet is completely dry before adding sample buffer, as any leftover TCA will turn the Bromophenol Blue in the sample buffer yellow.
9. The majority of the proteins secreted by filamentous fungi can have carbohydrate contents reaching up to 50% of the total molecular weight of the protein ***(1)***. This heavy glycosylation is thought to be responsible for the tendency of these proteins to show "smearing" on SDS-PAGE gels ***(12)***. Enzymatic protein deglycosylation using PNGase F or chemical deglycosylation using TFMS acid will help to obtain better resolution of the protein bands on the SDS-PAGE and 2-DE gels. Deglycosylation can also aid in identification of proteins by MALDI-MS by obtaining better-resolved peaks in the mass spectra.
10. The preparation of samples for gel electrophoresis requires prior knowledge of protein concentration to determine the amount of protein to be loaded. The protein concentration in the concentrated supernatant broths can be determined according to the method of Bradford ***(15)*** using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories, Inc., Hercules, CA), or any other commercially available kit. As fungal culture broths may contain phenolic compounds, it is important to note that many of these compounds interfere with several of the most popular methods for protein determination. It is suggested that protein concentration is determined following protein precipitation and resolubilization in SDS Reducing Buffer or 2-DE sample buffer. Unfortunately, several components of these buffers may also cause problems in the assessment of protein concentration. Two commercially available protein determination kits that can be used with samples prepared for electrophoresis techniques are 2-D Quant

Kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) and Advanced Protein Assay (Cytoskeleton, Inc., Denver, CO).

11. Resolubilization of the precipitated protein pellet in 2-DE buffer may require vortexing and/or sonication. It has been recently shown that adding 20–30 µL of 0.2*M* NaOH to the TCA precipitated pellet for 2 min, before adding the solubilization buffer, increases the amount of soluble protein in the sample buffer ***(16)***.

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