Chapter 18

Investigation of In Vivo Protein Interactions in *Aspergillus* Spores

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Abstract

Understanding in vivo protein–protein interactions is critical to dissect precise functions of the regulatory proteins of fungal secondary metabolites. As many fungi differentially produce a diverse array of secondary metabolites during their lifecycle, it is important to understand the cell-type specific regulation of secondary metabolism. However, due to the difficulty of sample preparation of biologically active proteins in fungal spores, protein–protein interaction studies have been generally restricted. While some outstanding studies revealed protein–protein interactions of selected regulators, including the *velvet* proteins in vegetative cells, a detailed protocol for investigating the protein–protein interactions in the fungal spores has not yet been reported. Here, we describe a working protocol for the purification and identification of interacting protein partners of the spores of *Aspergillus nidulans* employing the VelB protein as an example.

Key words: *Aspergillus*, Protein interaction, Novel regulator, FLAG-tag, Spores

1. Introduction

One of the most intriguing aspects of the fungal lifecycle is the production of secondary metabolites that are regulated by internal and external inputs (signals). Understanding the gene regulation mechanism of secondary metabolite production has been a long-term goal in fungal science, and a number of gene regulation mechanisms are now known (1–5). Recent studies have revealed key upstream regulators called the *velvet* regulators (VeA, VelB, VosA, and VelC) that not only govern morphological development but also regulate secondary metabolism in fungi (6–8). Moreover, LaeA has been shown to be a global regulator of secondary metabolism as it plays an essential role in the production of various secondary metabolites including sterigmatocystin, lovastatin,

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penicillin, gliotoxin, and hyphal pigments in *Aspergillus* (9). In the nucleus the LaeA protein interacts with VeA the founding member of the *velvet* proteins, and VeA interacts with VelB, forming a trimeric complex (6, 7). In addition, VelB can form a homodimer or heterodimer with VosA (8). Importantly, the formation of velvet–LaeA complexes are thought to be highly dynamic and the resulting complexes would control different sets of biological processes, e.g., trehalose biogenesis or secondary metabolism, depending on the developmental stages and cell types.

Understanding protein–protein interactions leads to an advanced understanding of biological functions of a given protein by providing *in vivo* biochemical evidence. Several studies have shown protein–protein interaction data in the vegetative fungal cells using S-tag or tandem affinity purification (TAP) tag (7, 8, 10). Fluorescence protein tagging also has been broadly used for the localization of proteins in cells (6). However, the protein–protein interaction studies in fungal spores have been highly restricted in due to the difficult steps involved in the sample preparation of biologically active proteins. Moreover, the methods mentioned above still have some disadvantages, including high level of nonspecific background affinity, and/or disrupting the activity of other native proteins and protein–protein interactions. Here, we describe a detailed protocol for protein–protein interactions in asexual (conidia) and sexual (ascospores) spores of *Aspergillus nidulans* using FLAG-tag. The VelB protein is used as an example.

2. Materials

2.1. Spore Harvest and Lysis

1. Spore harvest buffer: PBS with 0.02% Triton X-100. Dissolve the following in 800 ml distilled H_2O. Add 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4 and adjust pH to 7.4. Add 0.2 ml of Triton X-100. Adjust volume to 1 l with additional distilled H_2O. Sterilize by autoclaving.
2. Spore wash buffer: 1× PBS without Triton X-100.
3. Spore lysis buffer: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, 15 mM EGTA, 0.02% Triton X-100, 20% Glycerol, 1 mM PMSF, and 1× Protease inhibitor cocktail. Prepare working solution from stock solution by dilution (see Note 1).
4. PMSF (200 mM) stock solution: dissolve 0.035 g of PMSF in 1 ml of 100% ethanol. Store at −20°C.
5. Protease inhibitor cocktail (25×): Dissolve one tablet of Protease inhibitor cocktail (Roche Cat #116974981001) in 2 ml of distilled water.

6. Zirconia/silica bead (0.5 mm, Research Products International Corp, Cat No. 9834).
7. 2 ml Screw cap microcentrifuge tubes with O-ring cap.
8. Mini-Beadbeater (Biospec, Cat. No. 607).
9. Dounce homogenizer (Norman D Erway Glass).

2.2. Pull-Down Using Anti-FLAG M2 Affinity Agarose

1. Anti-FLAG: M2 affinity agarose (Sigma Cat #A2220) (see Note 2).
2. 4× SDS-PAGE sample buffer: 0.3 M Tris–HCl (pH 6.8), 10% SDS, 25% B-mercaptoethanol, 45% glycerol, 0.1% bromophenol blue.
3. Sarmix® MR1 tube rotator (Sarstedt).

2.3. SDS Polyacrylamide Gel Components

1. *N,N,N',N'*-Tetramethyl-ethylenediamine (TEMED) (Sigma, T9281). Store at 4°C.
2. Resolving gel: Mix 3.33 ml of 30% Acrylamide/Bis-acrylamide (37.5:1), 3.75 ml of 1 M Tris-HCl (pH 8.8), 2.8 ml of distilled water, 100 µl of 10% SDS, 50 µl of 10% Ammonium sulfate (APS), and 5 µl of TEMED.
3. Stacking gel: Mix 1.33 ml of 30% Acrylamide/Bis-acrylamide (37.5:1), 1.25 ml of 1 M Tris-HCl (pH 6.8), 7.3 ml of distilled water, 100 µl of 10% SDS, 50 µl of 10% APS, and 10 µl of TEMED.
4. APS: 10% solution in distilled H_2O.
5. SDS-PAGE gel running buffer (10×, 1 l): dissolve 30 g of Tris base, 144 g of glycine, 10 g of SDS and adjust volume to 1 l with additional distilled H_2O.
6. Coomassie staining buffer: Dissolve 1.0 g of Coomassie powder-Brilliant Blue R-250 (Sigma Chemical Company, St. Louis, MO, USA) in 500 ml of distilled water in 1 l beaker and add 400 ml of methanol and 100 ml of acetic acid.
7. Destaining solution: Mix 200 ml of methanol, 100 ml of acetic acid, and 700 ml of distilled water in 1 l baker.

3. Methods

3.1. Preparation of the Protein Samples from the Asexual Spores Under Native Conditions

1. Grow WT and FLAG-tagged strains in appropriate media. Typically five plates of 2-day cultures are enough for 2–3 binding experiments. Scrape spores using cell scrappers from the plate using 10 ml of spore harvest buffer and pass through miracloth to remove mycelia and to collect spores.

2. Centrifuge 10 min at 1,700×*g* at 4°C using HS-4 rotor (Sorval).
3. Remove the supernatant and add 10 ml of cold spore harvest buffer.
4. Sonicate on ice (use a sonicator equipped with a microtip). Burst spores for 300 s (50% pulse) at 100–150 W to remove rodlets (see Note 3).
5. Centrifuge 10 min at 1,700×*g* at 4°C using HS-4 rotor and remove the supernatant.
6. Resuspend the spores in 20 ml of cold spore wash buffer and centrifuge 10 min at 1,700×*g* at 4°C. Remove the supernatant and add 20 ml of cold spore wash buffer. Centrifuge 10 min at 1,700×*g* at 4°C and remove the supernatant.
7. Resuspend the spores in 2–3 ml of spore lysis buffer and transfer 1.5 ml sample into a 2 ml O-ring screw cap microcentrifuge tube.
8. Add 200–300 μl of zirconia/silica beads to the tubes and break the cells.
9. Break the cells for 1 min using a bead beater and then keep the tube on ice for 10 min to cool it down. Heat generated during bead beating will denature proteins. Break the cells for an additional minute.
10. Centrifuge the lysate at 10,000×*g* for 10 min at 4°C to precipitate the cell debris. Transfer the supernatant in a new tube and add 1.0 ml of spore lysis buffer to the cell debris pellet and resuspend.
11. Respin once at 10,000×*g* for 10 min and save the supernatant. Combine the supernatant in 2 ml of tube and centrifuge at 10,000×*g* for 20 min to remove cell debris.
12. Pipette the clear supernatant into a new 2 ml tube and store on ice.
13. Take 10 μl aliquots and measure protein concentration.
14. Take 30 μl aliquots from each, add 10 μl of 4× Sample Buffer, and store at –20°C.

3.2. Preparation of the Protein Samples from Ascospores Under Native Conditions

1. Grow the WT and FLAG-tagged strains in appropriate media. Grow 15 plates of 7-day culture (wrapped in aluminum foil) for one binding experiment. Scrape the asexual spores and cleistothecium from the plates using spore harvest buffer and pass through miracloth to remove asexual spores.
2. Scrape cleistothecium on the miracloth using a sterile spatula and resuspend in 10 ml of spore harvest buffer.
3. Centrifuge 5 min at 770×*g* at 4°C using HS-4 rotor (Sorval).
4. Remove the supernatant and add 10 ml of cold spore harvest buffer.

5. Break the cleistothecium using dounce homogenizer and pass through miracloth to remove mycelia and collect ascospores. You will get red ascospores (see Note 4).
6. Centrifuge 10 min at 1,700 × *g* at 4°C using HS-4 rotor and remove the supernatant.
7. Resuspend the ascospores in 1.5 ml of spore lysis buffer and transfer into a 2-ml O-ring screw cap microcentrifuge tube.
8. Add 200–300 µl of zirconia/silica beads to the tubes and break the cells.
9. Break the cells for 1 min using a bead beater and then keep the tube on ice for 10 min to cool it down. Heat generated during bead beating will denature proteins. Break the cells 1 more min.
10. Centrifuge the lysate at 10,000 × *g* for 10 min at 4°C to pellet cell debris. Save the supernatant in new tube and add 1.0 ml of spore lysis buffer and resuspend. Respin one time at 10,000 × *g* for 10 min and save the supernatant. Combine the supernatant in 2 ml of tube and centrifuge at 10,000 × *g* for 20 min to remove cell debris.
11. Pipette the clear supernatant into a new 2 ml tube and store on ice.
12. Take 30 µl aliquots from each, add 10 µl of 4× Sample Buffer, and store at –20°C.
13. Take 10 µl aliquots and measure protein concentration.

3.3. Pull-Down Using Anti-FLAG Affinity Gel Under Native Conditions

1. Add 40 µl of the 50% anti-FLAG M2 affinity agarose slurry to the cleared lysate (total of 10 mg protein) and incubate for 3 h in the cold room on the tube rotator (see Note 5).
2. After incubation, spin down anti-FLAG agarose 2 min at 1,500 × *g*.
3. Take 30 µl aliquots from the supernatant; add 10 µl of 4× Sample Buffer (Unbound fraction).
4. Add 1 ml of spore lysis buffer and incubate on the Nutator 5 min (Wash 1). Centrifuge 2 min at 1,500 × *g*.
5. Take 30 µl aliquots from the supernatant; add 10 µl of 4× Sample Buffer (Wash 1 fraction).
6. Add 1 ml of lysis buffer and incubate on the Nutator (Wash 2). Centrifuge 2 min at 1,500 × *g*.
7. Take 30 µl aliquots from the supernatant; add 10 µl of 4× Sample Buffer (Wash 2 fraction).
8. Add 1 ml of lysis buffer and incubate on the Nutator (Wash 3). Centrifuge 2 min at 1,500 × *g*.
9. Take 30 µl aliquots from the supernatant; add 10 µl of 4× Sample Buffer (Wash 3 fraction).

10. Add 40 μl of 4× sample buffer (Elution fraction).
11. Analyze by SDS-PAGE. Boil samples for 5 min and load 20 μl of samples (see Note 6).

3.4. 10% SDS-PAGE Gel Electrophoresis

1. To make two resolving gels, mix 3.33 ml of 30% acrylamide mix, 3.75 ml of 1 M Tris-HCl (pH 8.8), 2.8 ml of distilled water, 100 μl of 10% SDS, 50 μl of 10% APS, and 5 μl of TEMED in 15 ml of conical tube. Pour the gel solution in Bio-Rad mini gel with 1 mm spacer before acrylamide is solidified. Leave space for stacking gel and add about 200 μl of water saturated isobutanol to prevent gel contacting atmospheric oxygen and level the resolving gel. Let the resolving gel at least 15 min to be solidified. When the resolving gel is solidified, pour off the isobutanol and wash the gel surface with distilled water.
2. To prepare separating gel, mix 1.33 ml of 30% acrylamide mix, 1.25 ml of 1 M Tris-HCl (pH 6.8), 7.3 ml of distilled water, 100 μl of 10% SDS, 50 μl of 10% APS, and 10 μl of TEMED in 15 ml of conical tube. Insert comb (1 mm thickness) in the separating gel gently. Let the gel sit for at least 15 min to solidify.
3. Prepare protein samples to run by heating for 5 min at 95°C for 5 min and then centrifuge for 5 min at maximum speed.
4. Load the samples in the well of stacking gel (10 μl/well) and electrophorese at 15 mA/gel constantly, so if you run two gels use 30 mA until the blue dye front reaches the bottom of the separating gel.
5. After gel running, separate the gel plates using a spatula and submerge the gels in Coomassie stain for 30 min with gentle shaking to stain the protein bands.
6. Pour off Coomassie stain and rinse the gels with destaining solution.
7. Cut bands, which are specifically detected on FLAG-tagged lane using razor blades. Use razor blade for one band to prevent cross contamination of the protein samples.
8. Transfer the bands in 1.5 ml of tubes and analyze the samples using MALDI-TOF mass spectrometer.

4. Notes

1. Prepare fresh working solution each time as PMSF and protease inhibitor cocktail are unstable.
2. As the anti-FLAG affinity gel is stored in 50% glycerol with buffer, make sure to remove glycerol just prior to use and equilibrate it with the lysis buffer. To equilibrate the resins,

take 40 μl of resin and mix it with 1 ml of the lysis buffer, and then rotate on the shaker for 1 h. Centrifuge the resins at low speed (1,500 × *g*) for 2 min and remove the supernatant. Resuspend the resins in 40 μl of lysis buffer. Do not exceed 1,500 × *g*, it may break the agarose.

3. Because the fungal spore rodlet is very hydrophobic, it interrupts protein–protein interaction during binding experiments. Removing the rodlets in the spore surface is the most critical point of getting successful results.
4. It is not necessary to sonicate ascospores as they lack rodlet on the surface.
5. Do not incubate samples more than 3 h because protease inhibitor is no longer active in 3 h, resulting in protein degradation.
6. To find specific proteins that bind to the FLAG-tagged proteins, make sure to load WT samples to differentiate the bands that exist only on the FLAG-tagged fraction. If it can be detected visually in the Coomassie staining, the band can be analyzed by MALDI-TOF.

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