Proteomic Evaluation of the Spore and Hyphae Proteomes of *Alternaria alternata*

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Abstract

Alternaria alternata is associated with allergic respiratory diseases, which can be managed with allergen extract-based diagnostics and immunotherapy. It is not known how asexual spores, or vegetative hyphae contribute to patient allergen reactivity and commercial allergen extracts are manufactured by extracting proteins without separating these components. To better understand how allergens are distributed in *A. alternata* and to identify biological differences in the proteome, we performed data independent acquisition (DIA) mass spectrometry to quantitatively compare the proteomes of asexual spores (germinating and non-germinating) with vegetative hyphae. Collectively, quantitative proteomic analysis (N=6) identified 3638 proteins in all sample types and revealed that known *A. alternata* allergens were more abundant in non-germinating spores. When comparing non-germinating spore and hyphae proteomes directly, 174 proteins were reproducibly upregulated or specific to spores and 80 proteins were upregulated or specific to hyphae. Included among the 174 spore proteins were those functionally involved in cell wall synthesis, cellular stress, maintaining redox balance and homeostasis. Notably, one of the most abundant proteins in the spore proteome was a novel intracellular sialidase (neuraminidase) which may enhance allergen delivery in the lungs. When comparing both non-germinating and germinating spore proteomes, 25 proteins were reproducibly upregulated or specific to non-germinating spores and 54 were upregulated or unique to germinating spores. Among the proteins specific to germinating spores were proteases and other proteins known to be virulence factors associated with fungal pathogenicity. The data in this study provide new information that may be utilized for improving extract potency and specificity in commercial allergen extracts and also may shed light on the mechanism of allergen delivery in the lung epithelium.

Introduction

Alternaria alternata, a ubiquitous fungus, can elicit immunoglobulin E (IgE)-mediated respiratory diseases. Licensed commercial extracts used for immunotherapy are non-standardized, and the manufacture of more consistent extracts could enhance the diagnosis and treatment of Alternaria allergy. The life cycle of mold is complex and understanding the appearance of different allergens in different developmental stages will enhance manufacturing control over allergen content. To better understand the allergen profile and biological differences between different developmental stages within the mold life cycle, we performed comparative proteomic analyses of *Alternaria alternata* spores, germinating spores and hyphae utilizing quantitative mass spectrometry.

*Materials and Methods

Spore and hyphae protein extraction method (see Figure 1A): Spore, germinating spore and hyphae samples were prepared and isolated from *A. Alternaria* strain CBS 916.96. Each isolated sample type was first pulverized in a mortar and pestle (with liquid nitrogen), followed by protein extraction using acetonitrile buffer containing 2.5% trifluoroacetic acid. The resulting supernatants were collected, dried, washed with 90% acetone, and lyophilized and stored at -20 °C. Before mass spectrometry analysis, resuspended protein extract were quantified using a 2D quant assay (Cytiva), and then analyzed by SDS-PAGE to confirm suitable extraction and complexity for proteomic evaluation.

In-gel spore and hyphae proteomic workflow (see Figure 1B): For each study*, equivalent amounts of each sample type (in triplicate) were run on a 1D SDS-PAGE gel for 5 min, stained for 1 hour and then destained overnight. The resulting bands were cut out, cubed, placed in labeled tubes and then subjected to standard in-gel trypsin digestion overnight at 37 °C. **Comparative Quantitative Proteomics:** For each sample data acquisition, extracted peptides were loaded onto a reversed phase C18 column coupled online to an Orbitrap Lumos Tribrid mass spectrometer (Thermo) and then analyzed over a 2 hour elution gradient using data independent acquisition (DIA) analysis. Resulting MS/MS spectra were searched against the complete Uniprot *A. alternata* database (reviewed and unreviewed entries) using the software Protalizer (Vulcan Analytical). This software provides both identifications and fold change comparisons of proteins found in different samples. All fold changes are normalized to endogenous references seen in samples with similar retention time coordinates.

*This presentation is based on the evaluation of data collected from two independent studies. For each study, three separate non-germinating spore, germinating spore and hyphae replicates were analyzed (N=6 for each sample type).

Materials and Methods

Figure 1 A **Spore and hyphae protein extraction method**

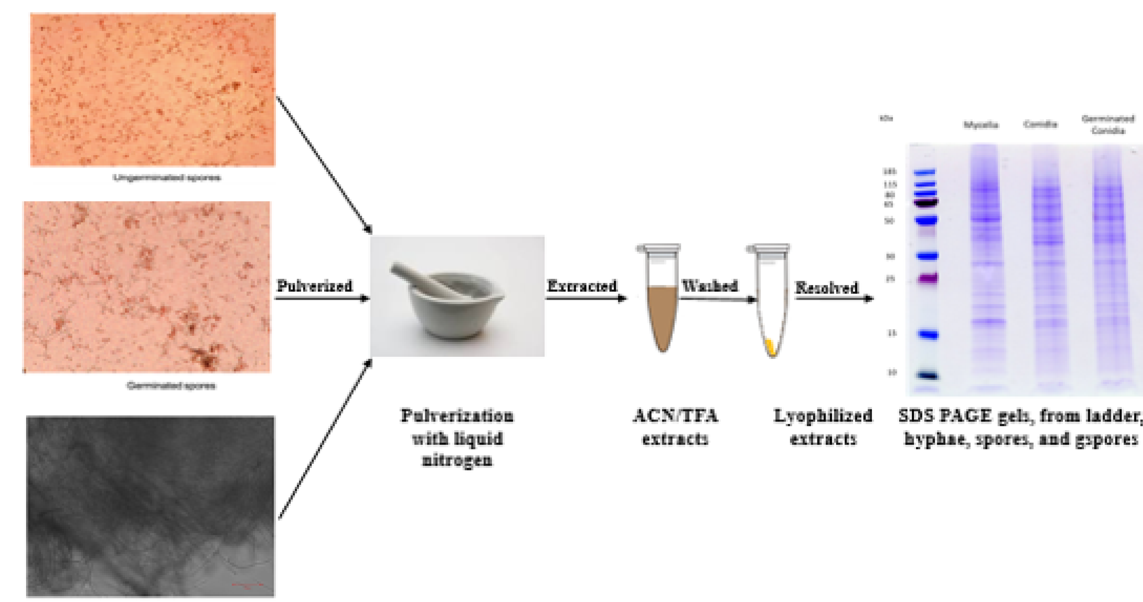

Figure 1B **In-gel spore and hyphae proteomic workflow**

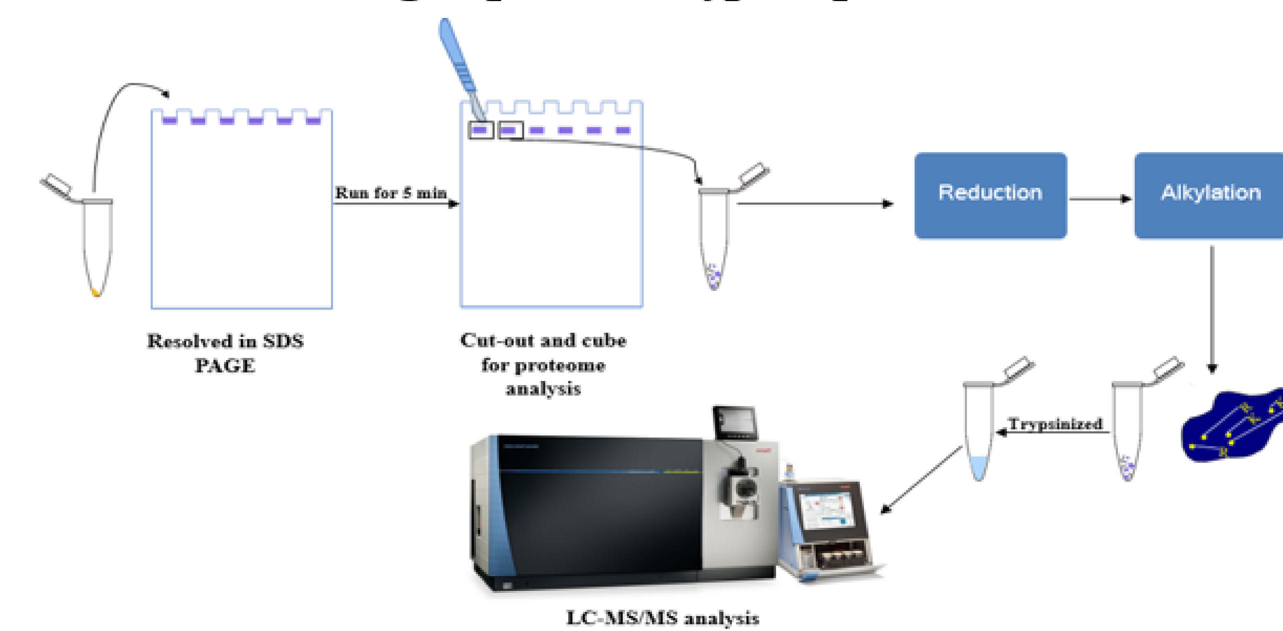

Figure 1. (A) Spore and hyphae extraction method: All sample types were pulverized with a mortar and pestle. Proteins from ungerminated spores, germinating spores, and hyphae, were extracted individually with acetonitrile/2.5% trifluoroacetic acid buffer and then lyophilized. Protein extracts were analyzed by SDS-PAGE to confirm suitable extraction and complexity for proteomic evaluation. **(B) In-gel spore and hyphae proteomic workflow:** Equivalent amounts of each sample to be analyzed were processed by in-gel trypsin digestion. Extracted peptides were loaded onto a 1D C18 column couple online and then analyzed over a 2 hour mobile phase gradient on an Orbitrap Lumos Tribrid mass spectrometer by data independent acquisition mode.

Results and Discussion

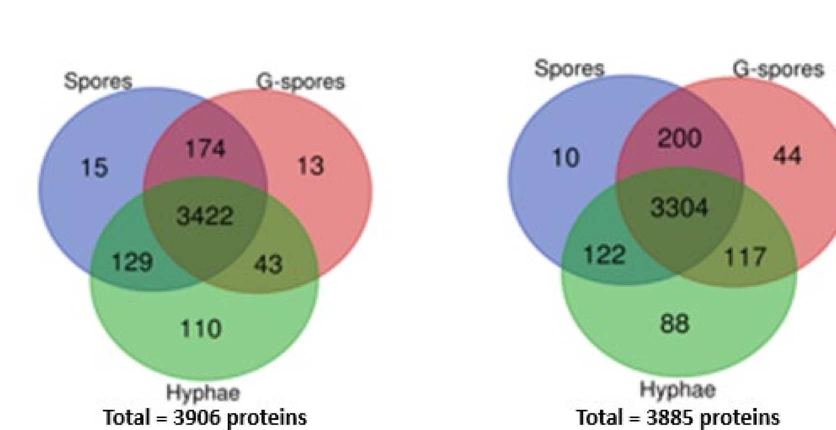

Figure 2. 1st Dataset: 3906 proteins were identified in all three samples. 3442 proteins were common in all three proteomes. 15 proteins were specific to spores, 13 proteins were specific to germinating spores and 110 proteins were specific to hyphae. **2nd Dataset:** 3885 proteins were identified in all three samples. 3304 proteins were common in all three proteomes. 10 proteins were specific to spores, 44 proteins were specific to germinating spores, and 88 proteins were specific to hyphae.

Results and Discussion

Figure 3. Allergen profile comparisons for spores versus hyphae

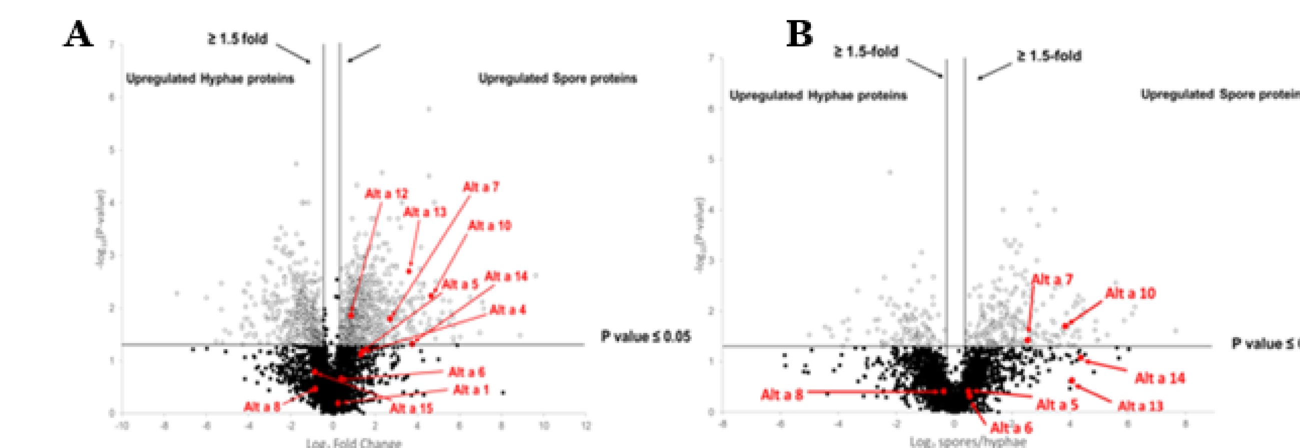

Figure 4. Allergen profile comparison for spores versus germ. spores

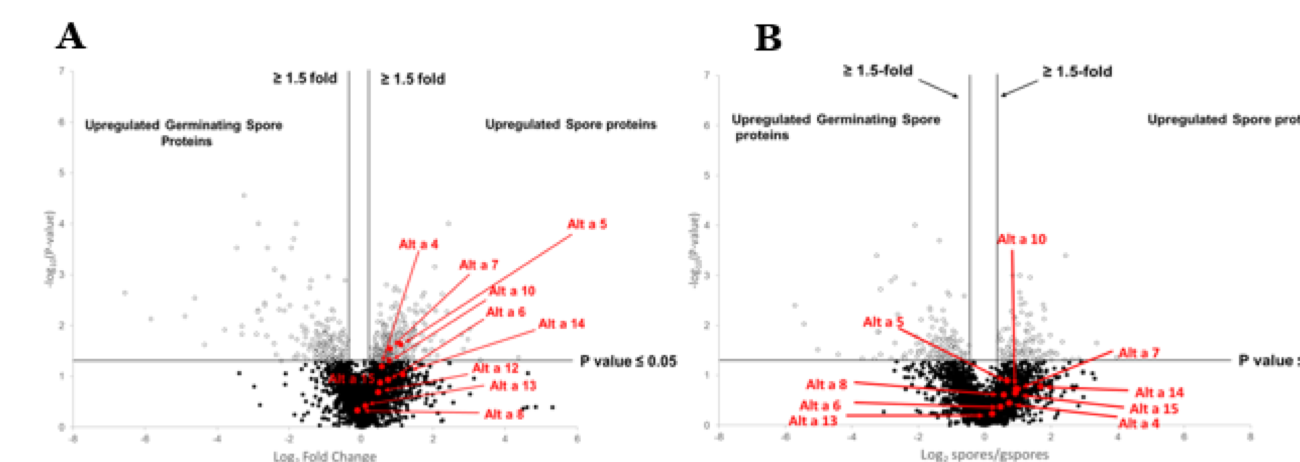

Figure 3. Allergen profile comparison for spores and hyphae. (A) 1st dataset, Volcano plot of 3502 *A. alternata* proteins present in both. The allergen Alt a 3 was only identified in spore. **(B)** 2nd dataset, Volcano plot of 3014 *A. alternata* proteins present in both. The major allergen Alt a 1 was only identified in hyphae.

Figure 4. Allergen profile comparison for spores and germinating spores. (A) 1st dataset, Volcano plot of 3261 *A. alternata* proteins. The major allergen Alt a 1 and 3 was only identified in spore. **(B)** 2nd dataset, Volcano plot of 3105 *A. alternata* proteins. The major allergen Alt a 1 was only identified in hyphae.

WHO known *A. alternata* allergens

Allergen	Biochemical name	Uniprot
Alt a 1	Major allergen Alt a 1	P79085
*Alt a 3	Heat Shock Protein 70	P78983
Alt a 4	Protein Disulfide-isomerase	Q00002
Alt a 5	Ribosomal Protein P2	P42037
Alt a 6	2-phosphoglycerate dehydratase (enolase)	Q9HDT3
Alt a 7	Minor allergen Alt a 7 YCP4 homolog	P42058
Alt a 8	NADP dependent mannitol dehydrogenase	P0C0Y4
Alt a 10	Aldehyde dehydrogenase	P42041
Alt a 12	60S acidic ribosomal protein P1	P49148
Alt a 13	Glutathione-S-transferase	Q6R4B4
Alt a 14	[Mn] Superoxide dismutase	P86254
Alt a 15	Subtilisin-like serine protease	A0A0F6N3V8

*Alt a 3 is not listed on volcano plots because it was only identified in spore samples

Figure 5. Spore and hyphae upregulated protein classes

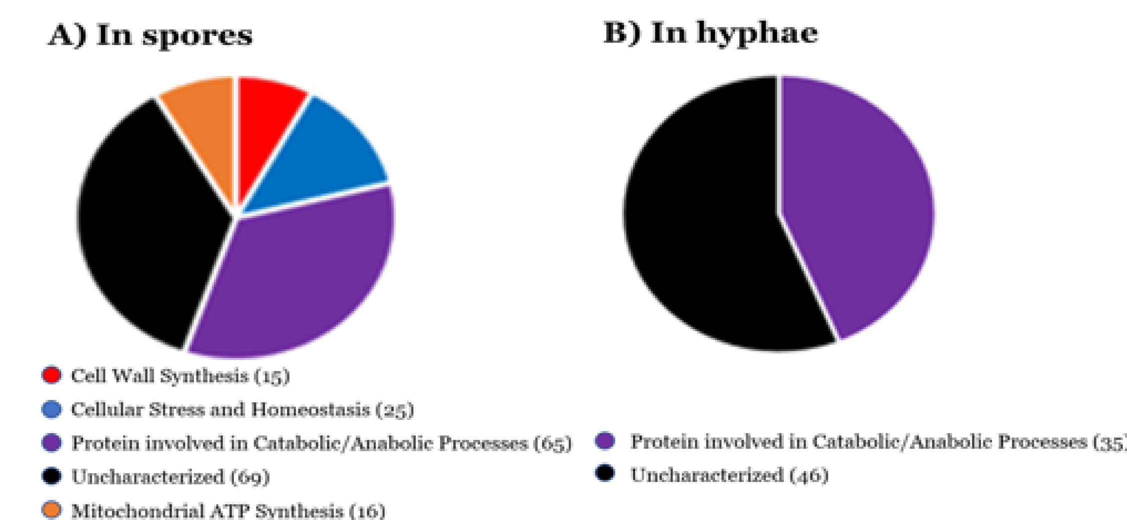

Figure 5. Upregulated (P<=0.05) proteins in both 1st and 2nd dataset (from direct comparison of spore and hyphae proteomes). (A) 174 proteins were upregulated or specific to spores. These fell into 5 classes including, cell wall synthesis (15), cellular stress and homeostasis (25), catabolic/anabolic process (65), mitochondrial ATP synthesis (16) and uncharacterized (69). **(B)** 81 proteins were upregulated or specific to hyphae. These fell into 2 classes including, catabolic/anabolic process (35) and uncharacterized (46). Sialidase was the most abundant protein in all spore samples **(see Figure 6)**

Figure 6. The novel sialidase identified in spores is intracellular

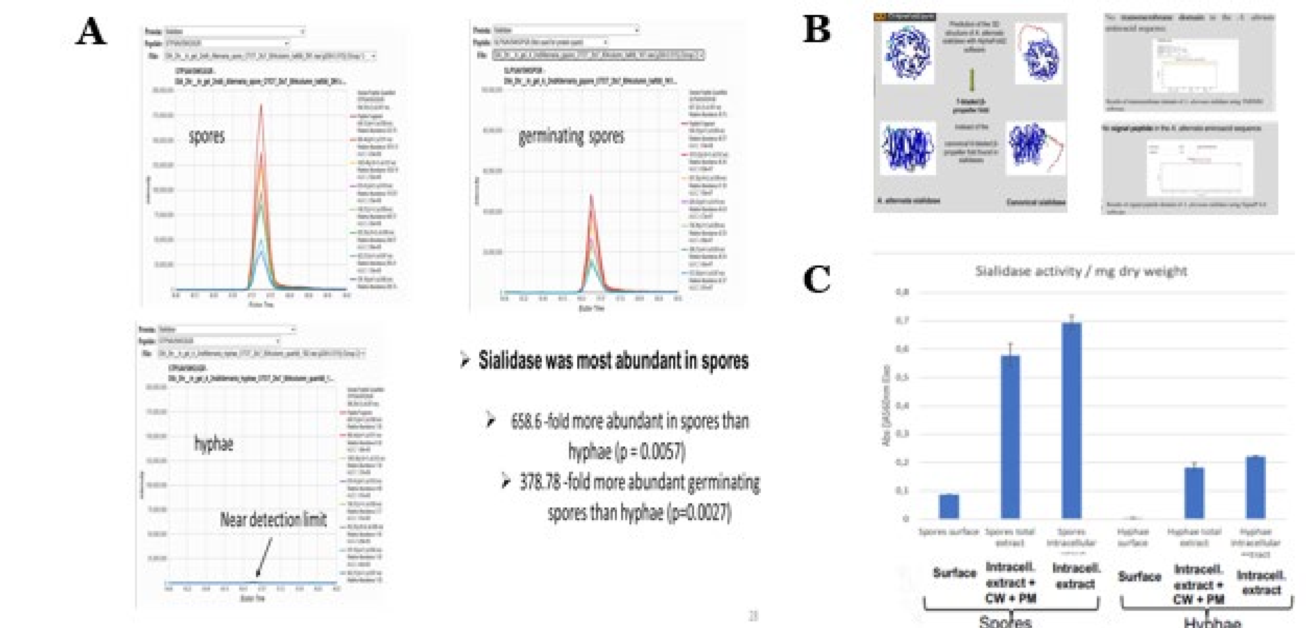

Figure 6. (A) Extracted ion chromatogram data for sialidase peptide used for quantification. This was the most abundant protein identified in spores based on ion current measurements. In contrast, this protein was barely detected in hyphae. **(B)** AlphaFold2 and TMHMM software both predict that this Sialidase has a novel 7 bladed β propeller fold that lacks both a transmembrane domain or signal sequence. **(C)** Sialidase activity were detected in both spore and hyphae samples, as an intracellular protein. This activity is more pronounced in spores.

Figure 7. Spore and germ. spores upregulated protein classes

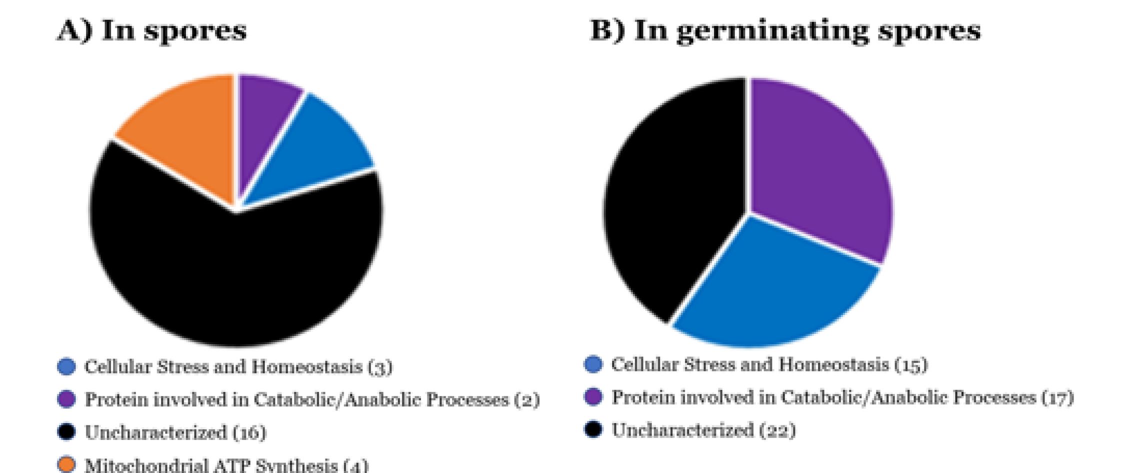

Figure 7. Upregulated (P<=0.05) in both 1st and 2nd dataset (from direct comparison of spore and germinating spore proteomes). (A) 25 proteins were upregulated or specific to spores. These fell into 4 classes including, cellular stress and homeostasis (3), catabolic/anabolic process (2), mitochondrial ATP synthesis (4) and uncharacterized (16). **(B)** 54 proteins were upregulated or specific to germinating spores. These fell into 3 classes including, cellular stress and homeostasis (15) catabolic/anabolic process (17) and uncharacterized (22). 9 out of the 15 proteins involved in regulating cellular stress and homeostasis are proteases or other proteins known to be virulence factors associated with fungal pathogenicity.

Conclusion

- Known *A. alternata* allergens are predominantly more abundant in the spores proteome (Figure 3 and 4).
- Among the 174 proteins reproducibly upregulated or specific to spores are proteins involved cell wall synthesis, and responding to cellular stress. The most abundant protein in spores is a novel sialidase (neuraminidase). This protein may be involved in allergen delivery in the lungs.
- The sialidase identified in spores is barely detected in hyphae (Figure 6). Structural prediction software suggest that this sialidase has a novel 7 bladed β propeller fold that lacks both a transmembrane domain or signal sequence. Preliminary sialidase assay data indicate that this protein is intracellular and most abundant in spores.
- 9 of the 15 proteins involved in regulating cellular stress and homeostasis in germinating spores are proteases and other proteins known to be virulence factors associated with fungal pathogenicity.

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