Proteomic evaluation of *Alternaria alternata* spores, hyphae, and commercial allergen extracts

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Abstract

Background and Rationale: *Alternaria alternata* is associated with allergic respiratory disease, which has led to the need for allergen extract-based immunotherapies and diagnostics. Available commercial Alternaria allergen extracts are neither standardized nor well-characterized with regard to allergen content. Immunotherapy and diagnosis with existing products, while safe and effective, could be improved with better characterization and manufacturing consistency. The goal of this study is to apply analytical methods, including quantitative mass spectrometry, for comparative and comprehensive characterization of Alternaria allergen extracts from various source materials.

Methods: Spore and hyphae preparations of *A. brassicacola* and *A. alternata* were prepared in various growth media and extracted under a variety of conditions. Extracts were then subjected to SDS-PAGE (one- and two-dimensional), and IgE-immunoblotting using human allergic sera. Using these approaches, our laboratory optimized extraction methods that are amenable to downstream comparative proteomics, which includes commercial *A. alternata* extracts.

Results: Extracts prepared from spores and hyphae had higher protein abundance, greater complexity and more IgE-reactivity than commercial extracts.

Conclusion: The preliminary results from our optimization studies lay the groundwork to perform in-depth comparative proteomic analyses using data independent acquisition liquid chromatography tandem mass spectrometry strategies. Our goal from these future studies will be to elucidate quantitative and qualitative differences between known and candidate allergens from spore and hyphae proteomes. We will then apply this information toward developing multiple reaction monitoring assays (a mass spectrometry-based assay) for absolute quantification of allergen content and standardization of Alternaria extracts.

Introduction

Alternaria alternata, a ubiquitous fungus, can elicit immunoglobulin E (IgE)-mediated respiratory diseases. Licensed commercial extracts used for immunotherapy are heterogeneous, and the manufacture of more consistent extracts will enhance the diagnosis and treatment of Alternaria allergy. The life cycle of molds is complex and understanding the appearance of different allergenic proteins in different developmental stages will further enhance manufacturing control over extract allergen content. This study aims to do a comparative proteomic analysis of *Alternaria alternata* spores and hyphae and characterize commercial allergenic extracts utilizing quantitative mass spectrometry. We also study spores and hyphae obtained from another Alternaria species, *A. brassicicola*.

Materials and Methods

A. alternata and *A. brassicicola* spore and hyphae proteins were extracted using acetonitrile buffer containing 2.5% trifluoroacetic acid [in volume ratio 7:3]. Supernatants were collected, dried, and washed with 90% acetone. Protein concentration of the extracts was determined using 2D quant assay (Cytiva), and the extracts were analyzed by SDS-PAGE and 2D electrophoresis. IgE immunoblotting was performed after transfer to PVDF using pooled sera from Alternaria-allergic individuals. For the preliminary comparative quantitative proteomic experiment, an Orbitrap Lumos Tribrid mass spectrometer (Thermo) was utilized to perform data independent acquisition (DIA) analysis (in triplicate) on trypsinized *A. brassicicola* spore and hyphae peptides (n=1) and *A. alternata* spore and hyphae (n=3). *A. alternata* data were searched against the complete Uniprot *A. alternata* database using the software Protalizer (Vulcan Analytical).

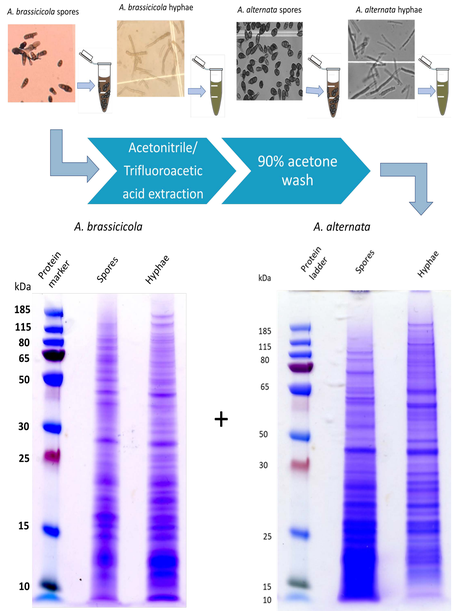


Figure 1: *A. brassicicola* and *A. alternata* spores and hyphae were extracted overnight using acetonitrile/2.5% trifluoroacetic acid. The supernatant was collected, dried and washed using 90% acetone. Post-extraction, proteins were quantified using 2D Quant Assay (Cytiva) and visualized via 1D SDS- PAGE.

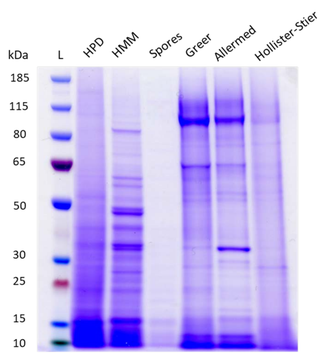


Figure 2. SDS PAGE comparing lab-made extracts to *A. alternata* commercial extracts. From left: protein ladder, lab-made *A. alternata* hyphae extract (PD broth), lab-made *A. alternata* hyphae extract (minimal media), lab-made *A. brassicicola* spores extract, Greer *A.alternata*, Allermed *A.alternata*, and Hollister-Stier, *A.alternata* extract. (Note that spores and hyphae, in this experiment, were extracted with conventional aqueous buffer).

Results and Discussion

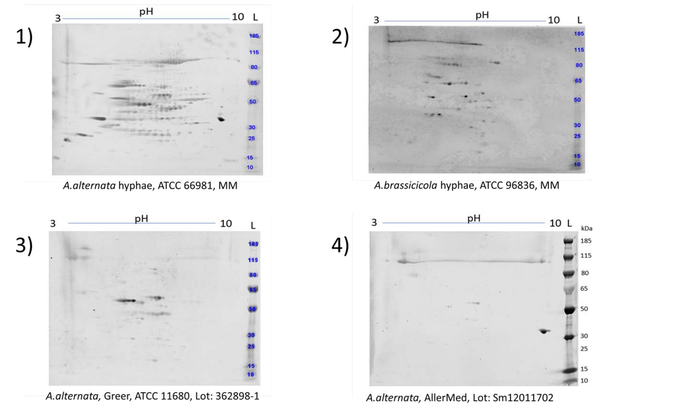
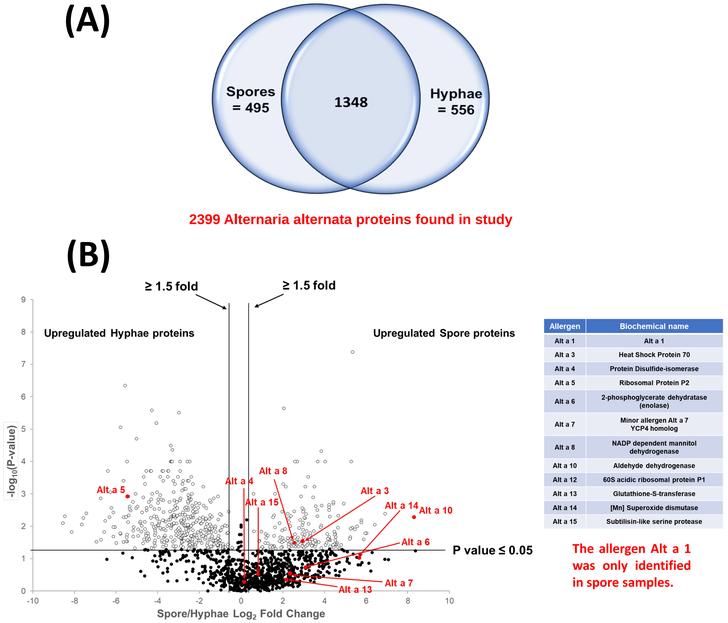


Figure 3. Western blots of lab-made *A. alternata* hyphae sample (1), lab-made *A. brassicicola* hyphae extract (2), and commercial *A. alternata* extract, Greer (3) and AllerMed (4) using *Alternaria* positive human sera.



Allergen	Biochemical name
Alt a 1	Alt a 1
Alt a 3	Heat Shock Protein 70
Alt a 4	Protein Disulfide-isomerase
Alt a 5	Ribosomal Protein P2
Alt a 6	2-phosphoglycerate dehydratase (enolase)
Alt a 7	Minor allergen Alt a 7 YCP4 homolog
Alt a 8	NADP dependent mannitol dehydrogenase
Alt a 10	Aldehyde dehydrogenase
Alt a 12	60S acidic ribosomal protein P1
Alt a 13	Glutathione-S-transferase
Alt a 14	[Mn] Superoxide dismutase
Alt a 15	Subtilisin-like serine protease

Figure 4. Proteome study employing 3 biological replicates from *A. alternata* spores and hyphae. **(A)** Venn Diagram representing *A. alternata* proteome results. **(B)** Volcano plot of 1348 proteins identified in both samples, showing allergen profile comparisons. Upper right quadrant represent spore upregulated proteins and upper left quadrant represent hyphae upregulated proteins. *A. alternata* allergens are highlighted (WHO/IACUC).

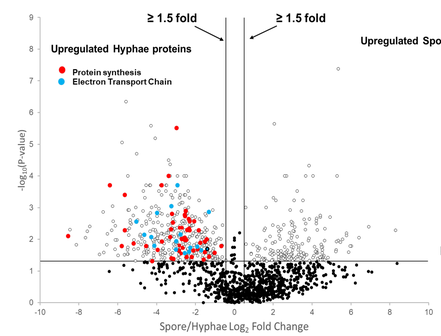


Figure 5. Volcano plot representing 1348 proteins identified in both *A. alternata* samples, showing gene ontological comparison of spores and hyphae for protein translation and electron transport chain components. Translational machinery including ribosomal proteins, translation initiation factors, elongation factors and signal recognition particle subunits were consistently upregulated in hyphae (highlighted in red). Mitochondrial proteins representing all 5 ETC complexes were upregulated in the hyphae (highlighted in blue).

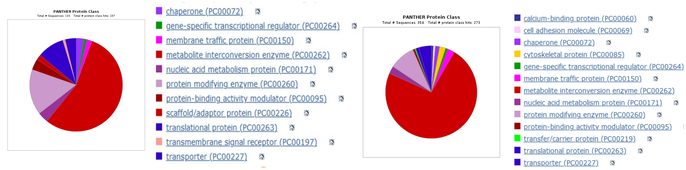


Figure 6. Because allergens are more prevalent in *A. alternata* spores (see Figure 4), we performed a gene ontological analysis to evaluate spore proteome. The pie charts demonstrate the distribution of major protein classes found to be upregulated and specific to the spore proteome. Large proportion of spore proteome represented metabolite interconversion enzymes (represented by the red sectors). Many of these enzymes include proteins responsible for regulating cellular stress and maintaining cellular homeostasis.

Conclusion

- Extracted spore and hyphae samples are different from each other (see Figure 1).
- The 2D western blots (Figure 3) show different levels of complexity depending on the sample source, confirming greater complexity for lab-made samples.
- Preliminary data show that spore and hyphae proteomes in *A. alternata* are distinct (Figure 4A).
- Commercial extracts are heterogenous and appear to be less complex than lab-made hyphae extracts (Figure 2).
  - Apart from Alt a 5 which is more abundant in hyphae, currently known allergens are consistently more abundant in spores (Figure 4B).
- Based on our initial gene ontological comparison of *A. alternata* spore and hyphae proteomes, protein translation and ATP synthesis are upregulated in the hyphae proteome (Figure 5).
- A large proportion of proteins upregulated or specific to spores are involved in responding to cellular stress and maintaining cellular homeostasis; many of the allergens linked to *Alternaria alternata* are in this category.
- Future experiments will evaluate the global PTM profile to see if there is a correlation with post-translational modification and biological pathway regulation.
- Multiple Reaction Monitoring assays using a Xevo TQ XS mass spectrometer (Waters) will be performed to quantify absolute concentration of allergens to better elucidate differences in the allergenic profiles of spores and hyphae.
- The information from these studies will ultimately be important toward improving allergen extract quality and standardization.

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