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Antigenic/Allergenic Analysis of Basidiomycete Cap, Mycelia, and Spore Extracts¹

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Abstract. Since airborne basidiospores may be important inducers of respiratory allergy, extracts of spores, caps and mycelia from *Pleurotus ostreatus* were studied by immunologic techniques. Crossed immunoelectrophoresis of the *Pleurotus* spore extract showed it to be a complex mixture containing at least 27 precipitating antigens. Crossed-line immunoelectrophoresis comparing *Pleurotus* spore extract with extracts of *Pleurotus* cap or mycelia demonstrated both common antigens and antigens unique to the spore extract. Inhibition of *Pleurotus* spore RAST by extracts of *Pleurotus* spore, cap, and mycelia suggested that these extracts contained common allergenic components. However, wheal and flare skin reactivity of allergic patients to extracts of *P. ostreatus* or *Cantharellus cibarius* demonstrated little correlation between reactivity to cap and spores. These results demonstrate that basidiomycete spore extracts are the best diagnostic reagents to use in clinical studies, although cap and mycelia extracts may provide useful material for further allergen analysis.

Introduction

Basidiomycetes, the most advanced of all fungal classes, number about 25,000 species and contain many diverse forms, including mushrooms, puffballs, rusts, smuts, and bracket fungi. Since atmospheric basidiospore concentrations are high in many parts of the world [1-3], and have been shown to induce positive skin reactions in patients with respiratory allergies [4-10], they may be of great importance in provoking respiratory allergic symptoms [11].

Pleurotus ostreatus is a wood-rotting gilled mushroom found both in Europe and North America [12]. It is of particular interest from an allergic standpoint, as commercial cultivation of the fungus has been associated with significant occupational respiratory allergy [13]. Previous studies in this laboratory have demonstrated that extracts prepared from mycelia or culture fluid of *Pleurotus* grown in vitro were allergenic in man and antigenic in the rabbit [14]. Furthermore, the prevalence of skin prick test reactivity to

Pleurotus as well as other basidiospore extracts was shown to be similar to that for other more commonly recognized fungal aeroallergens [10].

Because natural exposure occurs to airborne spores, spore extracts appear to be more relevant as compared to other basidiomycete components in the study of basidiomycete-induced respiratory allergy [15]. Unfortunately, such extracts are expensive and difficult to prepare. For this reason, the antigenic and allergenic properties of *Pleurotus* spore were compared with *Pleurotus* cap and mycelial extracts. Our findings indicate that antigenic/allergenic similarities exist, suggesting the potential use of mycelial or cap extracts as source material for purified allergenic extracts. However, use of crude extracts other than spores in clinical diagnostic studies is not recommended since significant differences were detected in cap and spore skin prick test reactivity.

Materials and Methods

Fungal Extracts

P. ostreatus, obtained from American Type Culture Collection (ATCC No. 9415), was cultured in vitro as previously described

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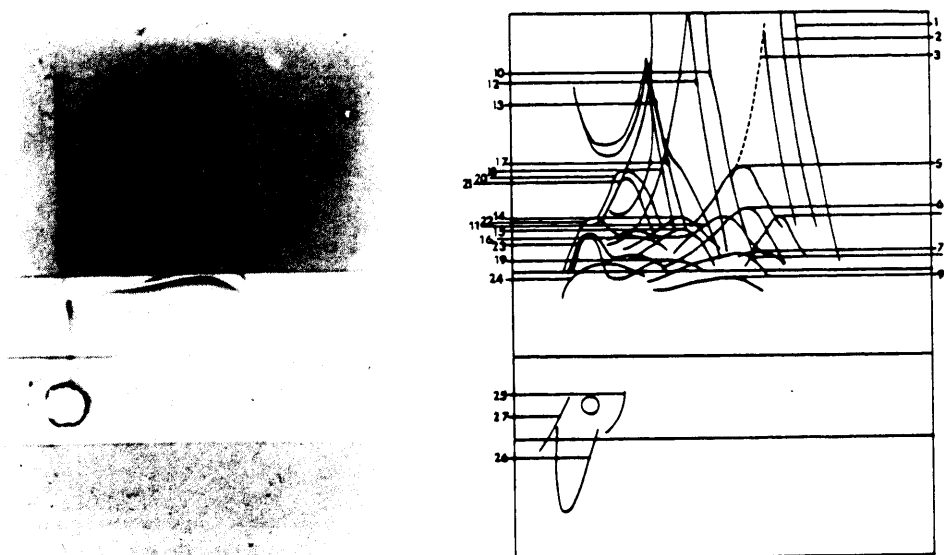


Fig. 1. *Pleurotus* spore extract examined by CIE. Specific rabbits' antisera demonstrated 27 distinct precipitin lines (24 anodic-migrating and 3 cathodic-migrating).

[14]. Briefly, cultures were grown in dialyzable yeast malt broth and mycelial growth harvested by filtration (Whatman No. 1 filter paper). The filtrate was dialyzed (Union Carbide; 18 FO MWCO 8–12,000) against H₂O and lyophilized (metabolic fraction). The remaining mycelial material was washed with ion-depleted water and lyophilized. Extracts were prepared by homogenization (Braun Homogenizer; 4,000 rpm, 2 min) of 1 g lyophilized mycelial growth in 20 ml carbonate buffer (pH 8.1), centrifuged (70,000 g), and the supernatant lyophilized (somatic fraction).

P. ostreatus, *Cantharellus cibarius*, *Ganoderma lucidum* and *Calvatia cyathiformis* spores were harvested as previously described [10, 15, 16]. Spores were extracted in carbonate buffer as described for mycelial growth. Following spore harvest, *Pleurotus* and *Cantharellus* caps were frozen and lyophilized. Lyophilized caps were ground to a powder (mortar and pestle) and extracted in carbonate buffer as described for mycelial growth.

Extracts of *Penicillium notatum*, *Aspergillus niger*, *Alternaria tenuis* and *Fusarium solani* were prepared from mycelial material (Greer Laboratories). Lyophilized mycelial material was sonicated in phosphate-buffered saline (PBS), pH 7.2, extracted at 4°C overnight, centrifuged, supernatants concentrated (Amicon UM 2), and frozen (–20°C) until used. Peanut extract was prepared from peanuts homogenized in PBS (100 g/l), extracted overnight at 4°C, centrifuged (70,000 g), and the supernatant concentrated (Amicon YM 2) aliquoted and frozen (–20°C) until used.

Immunoelectrophoretic Techniques

Antisera against *Pleurotus* spores were raised in 6 New Zealand white rabbits immunized 5 times with 10 mg of either *Pleurotus* spore homogenate or spore extract in complete Freund's adjuvant at 2-week intervals. Sera were collected weekly for 5 weeks, pooled, and concentrated by (NH₄)₂SO₄ precipitation (40% saturation) and following exhaustive dialysis against PBS concentrated by ultrafiltration (Amicon XM 50).

Crossed immunoelectrophoresis (CIE) and crossed-line immu-

noelectrophoresis (CLIE) were performed as described [17, 18]. Separation was performed on 5.9 × 6.3 cm plates of 1% agarose in barbituric acid buffer (pH 8.6). *Pleurotus* spore extract (240 µg) was applied in the well. First- and second-dimension separations were done at 10 V/cm for 38 min and 4 V/cm for 15 h, respectively. For CLIE studies, 2 mg of test extract was added to the intermediate gel [18].

RAST and RAST Inhibition

RAST and RAST inhibition were performed as previously described [19]. Antigen (10 mg/ml) in 0.1 M borate buffer (pH 8) was bound to cyanogen bromide-activated cellulose discs [20]. Serum (100 µl) was incubated with the discs in duplicate overnight, discs washed, and ¹²⁵I-labelled anti-human IgE (approximately 25,000 cpm; Pharmacia) added. The discs were again washed and cpm determined in a gamma counter, mean cpm calculated and expressed as percent binding of total radioactivity added.

RAST inhibition was performed in duplicate by incubating antigen-coated discs with 50 µl serum pool (5 sera from skin test positive subjects) plus 50 µl containing increasing concentrations of test antigen. The rest of the procedure was the same as that described for RAST. Results were expressed as percent inhibition calculated as follows: $[1 - \text{cpm test antigen} \div \text{cpm PBS control}] \times 100\%$.

Skin Prick Testing

Sterile cap or spore extracts of *Pleurotus* or *Cantharellus* at 5 mg/ml in 50% glycerol-PBS were used to skin prick test subjects with symptoms of respiratory allergy. Fifteen minutes later, wheals were measured, and a mean wheal diameter (largest diameter and one perpendicular to it) 2 mm or more greater than the negative control was considered positive. This dose of spore or cap extract was shown to be skin test negative (was not irritative) when tested in nonatopic subjects with no symptoms of respiratory allergy. Histamine diphosphate (1 mg/ml) and PBS-glycerol served as positive and negative controls respectively.

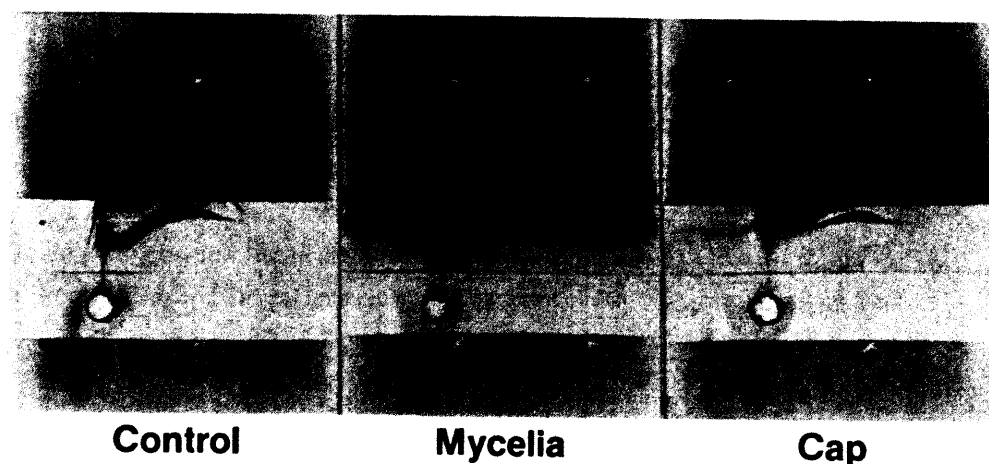


Fig. 2. Comparison of *Pleurotus* spore antigens with mycelia (somatic) or cap antigens by CLIE. When somatic or cap extracts were used as interacting antigens on these plates, both common antigens and antigens specific to the spore extract were demonstrated.

Table I. *P. ostreatus* spore antigens: comparison to mycelial or cap antigens by CLIE

CLIE test extracts	<i>P. ostreatus</i> spore precipitins (CIE)																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Micelia	-	-	-	4	5	6	7	-	-	-	-	12	13	14	-	16	17	-	-	20	21	22	23	24	25	26	-
Cap	-	-	-	4	5	6	7	-	-	10	-	12	13	-	15	16	17	-	-	20	21	22	23	24	25	26	-

Results

CIE and CLIE Analysis of *Pleurotus* Spore Extract

CIE analysis of the *Pleurotus* spore extract (using rabbit antisera) demonstrated 27 distinct precipitin lines, 24 anodic-migrating and 3 cathodic-migrating (fig. 1). Staining intensity of the precipitin lines varied widely with some antigens forming dense precipitin lines and others forming only very faint lines.

The antigenic constituents of *Pleurotus* spore extract were compared to those present in *Pleurotus* mycelia or cap extracts by CLIE (fig. 2). Some antigens in the *Pleurotus* spore extract showed immunologic identity with interacting antigens present in *Pleurotus* mycelia or cap extracts, and others did not (table I). Sixteen of the 27 antigens found in spore extract showed immunologic identity with constituents of mycelia extract, and 17 of the 27 antigens interacted with constituents of cap extract. The remaining spore antigens showed no interaction with mycelia or cap extracts by CLIE.

Inhibition of *Pleurotus* Spore RAST with *Pleurotus* and Other Fungal Extracts

The ability of different *Pleurotus* extracts to inhibit *Pleurotus* spore RAST was tested. As shown in figure 3a, *Pleurotus* cap and somatic extracts were potent inhibitors of *Pleurotus* spore RAST, inhibiting the RAST as effectively as spore extract itself. On the other hand, *Pleurotus* metabolic fraction was a relatively poor inhibitor of spore RAST. Control peanut RAST (fig. 3b) was essentially uninhibited by *Pleurotus* extracts. As shown in figure 4, *Pleurotus* spore RAST was not effectively inhibited by extracts of several *Fungi Imperfecti* species or by a basidiospore extract prepared from spores of *G. lucidum*. Minimal inhibition of *Pleurotus* spore RAST was observed with an extract of spores from *C. cyathiformis*.

Comparison of Skin Test Reactivity of *Basidiomycete* Spore and Cap Extracts

Since in vitro studies suggested antigenic/allergic similarities of cap and spore, extracts of both

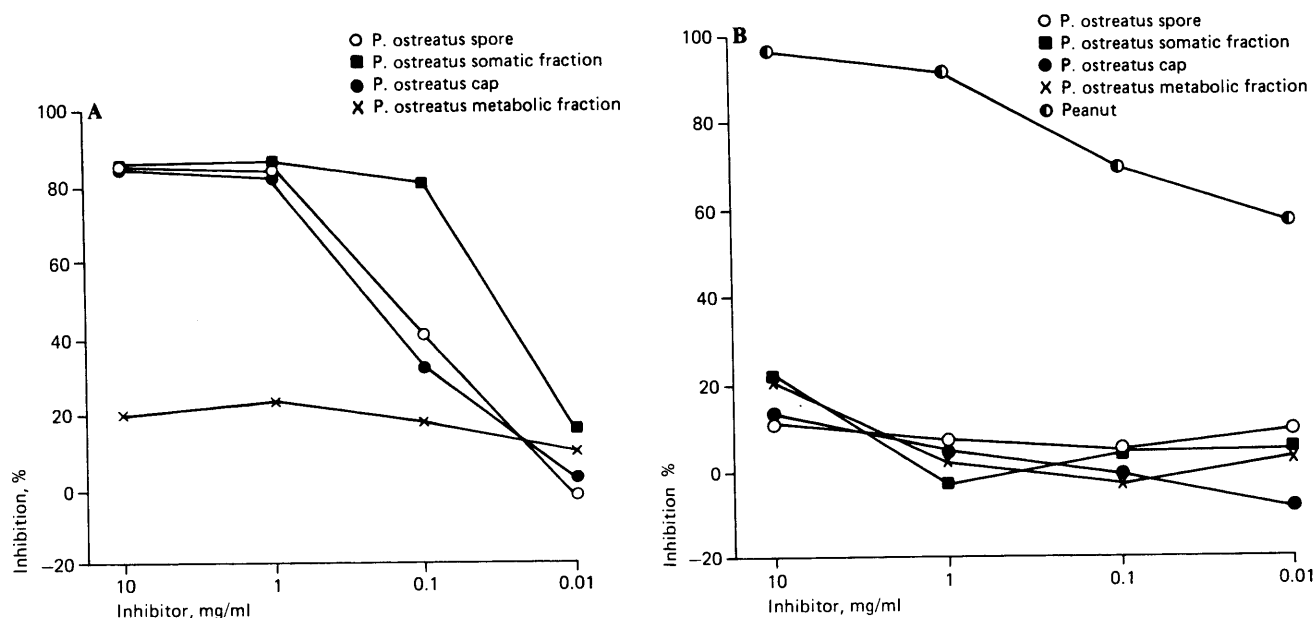


Fig. 3. A Inhibition of *Pleurotus* spore RAST with *Pleurotus* fractions. RAST discs were coated with *Pleurotus* spore extract. The discs were incubated with 50 μl containing increasing concentrations of test extract or PBS (positive control) and 50 μl of pooled RAST-positive sera (5 patients). Results are expressed as percent inhibition of ^{125}I -labelled anti-IgE binding to RAST discs, as compared to PBS control. **B** Inhibition studies of peanut RAST with *Pleurotus* fractions. RAST discs were coated with peanut extract. The discs were incubated with 50 μl containing increasing concentrations of test extract or PBS (positive control) and 50 μl peanut RAST-positive sera. Results are expressed as percent inhibition of ^{125}I -labelled anti-IgE binding to RAST discs, as compared to PBS control.

were tested *in vivo*. One hundred and four individuals who presented to the Tulane Allergy Clinic with rhinitis and/or asthma were skin prick tested with extracts of *Pleurotus* or *Cantharellus* cap or spores. Results, summarized in figure 5, demonstrate that 16 reacted to *Pleurotus* or *Cantharellus* extracts. Of these reactions, 7/16 reacted to both *Pleurotus* spore and cap extracts whereas only 2/16 reacted to both *Cantharellus* spore and cap extracts.

Discussion

Basidiomycetes have been implicated as potential inducers of respiratory allergy for many years [1-11]. Unfortunately, systematic study of this problem has been limited by a lack of well-characterized allergenic extracts [11]. Therefore, the antigenic and allergenic constituents of *Pleurotus* spore extract were analyzed in comparison to those of cap or mycelial (somatic) derived extracts.

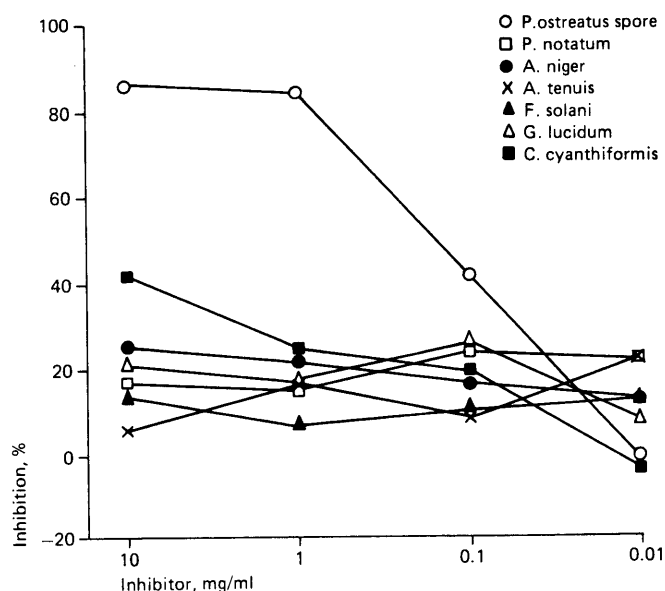


Fig. 4. Inhibition studies of *Pleurotus* spore RAST with fungal extracts. RAST discs were coated with *Pleurotus* spore extract. The discs were incubated with 50 μl containing increasing concentrations of fungal extract or PBS (positive control) and 50 μl of pooled RAST-positive sera (5 patients). Results are expressed as percent inhibition of ^{125}I -labelled anti-IgE binding to RAST discs, as compared to PBS control.

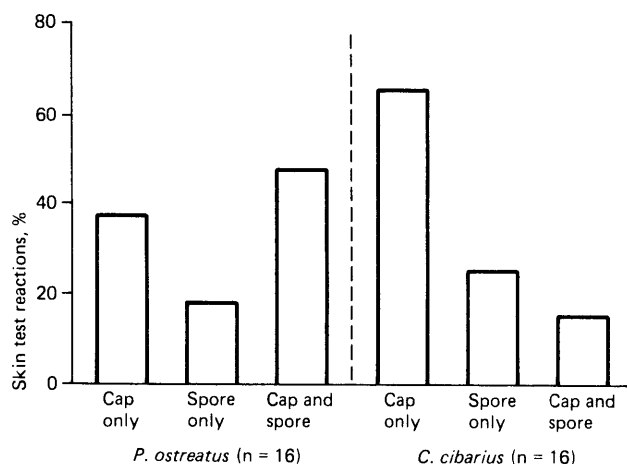


Fig. 5. Skin test reactions to basidiomycete cap and/or spore extracts. Of 104 subjects skin prick tested, 16 reacted to one or more fractions of *P. ostreatus* and 16 reacted to one or more fractions of *C. cibarius*.

CIE analysis showed the *Pleurotus* spore extract to be a complex mixture of antigenic components, as has been described in other mold extracts [21]. Although CLIE analysis showed that many antigenic constituents of *Pleurotus* spore extract shared determinants with those found in cap or somatic extracts, many of the spore antigens were unique to spore extract. The amount of cross-antigenicity was not surprising in the case of cap extracts (which contained spores), but was somewhat unexpected for the somatic extract obtained from spore-free mycelia.

RAST inhibition studies suggest that allergens present in *Pleurotus* spore, somatic, and cap extracts share common structures. Although this finding suggests that non-spore elements of *Pleurotus* might be useful source material for allergenic extracts, further analysis of skin test activity of spore and cap extracts from *Pleurotus* and *Cantharellus* did not generally support this. Less than 50% of subjects reacting to *Pleurotus* reacted to both cap and spores, and only 12.5% of *Cantharellus* skin test positive subjects reacted to both cap and spores. Furthermore, in the direct RAST assay, spore extracts were superior to those of cap [Lehrer and McCants, unpubl. data] or mycelia [22]. These results suggest that in spite of the antigenic/allergenic relationship of cap and spore extracts, significant differences remain when these ex-

tracts are used in clinical diagnostic testing. These results are similar to those reported earlier comparing *Pleurotus* metabolic, somatic and cap extracts with spore extracts by skin prick testing and reactivity with rabbit antisera [23]. However, in the previous study, we observed less reactivity to cap and somatic extracts as compared to our current study. The fact that these extracts are more active in this study is probably due to our current use of a more vigorous extraction procedure (as used for spores), yielding more active extracts.

The observed differences in allergen activity may reflect quantitative or even qualitative differences in allergen content. On a quantitative basis, if allergens in the *Pleurotus* spore extract constitute a major percentage of the proteins present, they would bind more effectively to cyanogen bromide-activated RAST discs, as compared to allergens present in cap or somatic extracts, if they are minor protein components. The lack of agreement with cap and spore skin test results may reflect qualitative differences in allergen content. Perhaps caps from *Pleurotus* or *Cantharellus* contain additional allergens that can sensitize directly (both are edible mushrooms) or cross-react with other vegetable products. Thus, further analysis of different sources of spore allergens by quantitative and qualitative methods of allergen analysis are necessary to measure basidiomycete/spore allergens from different sources.

It has been suggested that cross-allergenicity exists among members of some fungal species [24]. RAST inhibition studies did not confirm extensive cross-allergenicity between *Pleurotus* spore allergens and several members of the Fungi Imperfecti or basidiospores from *G. lucidum*. Moderate inhibition of *Pleurotus* spore RAST by an extract of *C. cyathiformis* basidiospores was observed, and suggests that partial cross-allergenicity may exist between spores of some species of basidiomycetes. This has recently been supported by correlation analysis of skin test and RAST reactions [25] and further RAST inhibition studies [26].

Overall, comparative studies of several sources of *Pleurotus* allergen for antigenicity in rabbits (CLIE) and allergenicity in man (RAST inhibition and skin test) indicate that somatic or cap-derived extracts may prove useful as source material for allergen purification and characterization. At present, however, such extracts should not be used in the diagnosis and management of allergy to inhaled basidiospores.

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