

Basidiospore allergens: analysis of *Coprinus quadrifidus* spore, cap, and stalk extracts

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Summary

Atopic individuals with symptoms of respiratory allergy have been shown to have IgE-mediated reactions to spores from the basidiomycete fungi. Because our earlier studies suggested that parts of the fungus other than spores may contain allergens, the current study was performed. Extracts of *Coprinus quadrifidus* spores, caps, and stalks were prepared and fractionated by gel filtration column chromatography on Sephadex G-75. Analysis of column fractions of each separation by ultraviolet absorption demonstrated at least three peaks of absorbency in spore, cap, and stalk extracts. Pooled column fractions were analysed by direct radio-allergosorbent test (RAST) using pooled sera from *C. quadrifidus* skin-test positive subjects. Enhanced allergenic activity was present in the same portion of the column eluate for cap, spore, and stalk fractionations, corresponding to a molecular weight of approximately 10.5–25 kD. Pools with allergenic activity were used to test volunteers by skin prick and RAST. Skin test and RAST activities were similar for each of the three *Coprinus* extracts, with stalk being the most potent. Evidence of common allergenic epitopes was demonstrated by inhibition of spore RAST by spore, cap, and stalk extracts. These results suggest that *C. quadrifidus* cap and stalk extracts contain allergens similar to those in spores extract and may provide useful sources of allergen for further study.

Introduction

Basidiomycetes are the most morphologically complex fungal class, numbering approximately 25 000 species. The class includes diverse forms such as mushrooms, puffballs, rusts, smuts, and bracket fungi [1]. Basidiospores were first reported to cause respiratory symptoms in 1951 [2]. Ten years later, increased levels of basidiospores were associated with exacerbations of 'epidemic asthma' in New Orleans [3]. Spores from basidiomycetes have been found in high concentrations in many parts of the

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world and are known to induce positive skin-test reactivity [4-8] or skin-test and bronchial reactivity [9,10] in subjects with respiratory allergy.

Coprinus, known as the inky cap mushroom, is a common basidiomycete genus, and is characterized by rapid autolysis of the mature fruiting body [11]. In the northern hemisphere, its fruiting season begins in the spring and usually continues through the fall [12]. In New Orleans, one of the species, *Coprinus quadrifidus*, may even be found fruiting in the winter.

Previous studies have shown *C. quadrifidus* spores to be significant aeroallergens in our area, as demonstrated by positive skin-prick test and RAST reactivity [8, 13]. Large quantities of mushrooms are required to provide sufficient quantities of spores to prepare even the smallest amount of extract. Preliminary evidence for another basidiomycete, *Pleurotus ostreatus*, showed that different parts of the mushroom may contain similar allergens and, therefore, may provide an additional source of allergen for extract preparation [14]. The aim of this study was to analyse allergens present in *C. quadrifidus* spores, and to compare spore allergens with those found in the cap and stalk by skin-prick test, RAST, and RAST inhibition.

Materials and methods

Extract preparation

Coprinus quadrifidus mushrooms were collected and separated into caps and stalks. The majority of caps (approximately 70%) were placed upright on filter paper for 4-8 hr. When autolysis began, the caps were removed and the spores were washed off the filter paper with deionized water, and lyophilized. The remaining caps (approximately 30%) and all of the stalks were immediately frozen overnight at -20°C , and lyophilized. The lyophilized caps and stalks were pulverized in a Waring blender for 2 min. All spores, powdered cap and powdered stalk, were stored under desiccation until extracted. For extraction, 1 g of *C. quadrifidus* caps, spores, or stalks were homogenized (Braun Homogenizer, 4000 r.p.m.) in 20 ml of 0.125 M ammonium bicarbonate buffer for 2 min. The homogenate was centrifuged at $80\,000\times g$ for 30 min, and the supernatant lyophilized.

Gel filtration

Blue dextran (2000 kD), ovalbumin (44 kD), and cytochrome-C (12.4 kD) were used as molecular weight markers. One to three grams of each of the extracts were resuspended in 10 ml of 0.125 M ammonium bicarbonate and centrifuged at $8000\times g$ for 5 min. Supernatants were then fractionated using a G-75 Sephadex column ($5.0\times 90\text{ cm}$) equilibrated with 0.125 M ammonium bicarbonate buffer. Ten-millilitre column fractions were collected drop-wise and optical density (280 nm) determined. Based on spectrophotometry, five pools (1-5) were prepared, lyophilized, and used for skin-prick testing and RAST.

Patients and skin tests

Sterile extracts in glycerol-PBS of *C. quadrifidus* spore, cap, and stalk combined column pools 3 and 4 were prepared at concentrations of 10, 1.0, 0.1, and 0.01 mg/ml. *Coprinus* extracts and twelve common inhalant allergen extracts were used to skin-prick test twenty-eight volunteers of whom twenty-four had a personal and/or family history of atopic disease.

Results were recorded at 20 min and considered positive if the mean weal diameter was at least 2 mm greater than the negative control (PBS). Histamine diphosphate (1 mg/ml) was used as the positive control. Sera were obtained for radio-allergosorbent tests (RAST).

Radio-allergosorbent tests

Radio-allergosorbent tests and RAST inhibition were performed as previously described [15]. Briefly, cyanogen bromide-activated cellulose discs were coupled overnight with combined pools 3 and 4 of spore, cap, or stalk at 10–20 mg/ml in borate buffer by the method of Ceska [16]. Serum (100 μ l) was incubated with the discs in duplicate overnight. Discs were washed and ^{125} I-anti-human IgE added at approximately 25 000 c.p.m. The discs were again washed and c.p.m. determined in a gamma counter, mean c.p.m. calculated, and results of individual serum samples expressed as per cent binding of total radioactivity added.

Radio-allergosorbent test inhibition was performed by incubating spore antigen-coated discs in duplicate with 50 μ l of pooled serum from five skin-test positive patients, plus 50 μ l of cap, spore, or stalk extract at increasing concentrations (0.001–10 mg/ml). Phosphate buffered saline was used as a negative control. The rest of the procedure was the same as that described for RAST. Results of RAST inhibition were recorded as per cent inhibition of the results obtained with RAST–PBS control.

Results

Spores, cap, and stalk extracts were prepared and fractionated on a G-75 Sephadex column. Figs 1–3 demonstrated the ultraviolet (UV) absorption patterns for each. As can be seen from these figures, at least three peaks of UV-absorbing material were demonstrated in the spore, cap and stalk extracts. Based on the spectrophotometry patterns, five pools of column fractions were prepared as indicated, and RAST activity determined for each. Maximum RAST activity was present in pools 3 and 4. These pools were combined and used in further testing.

Twenty-eight volunteers of whom twenty-four had a personal and/or family history of atopic disease were skin tested with twelve common inhalants and *Coprinus*

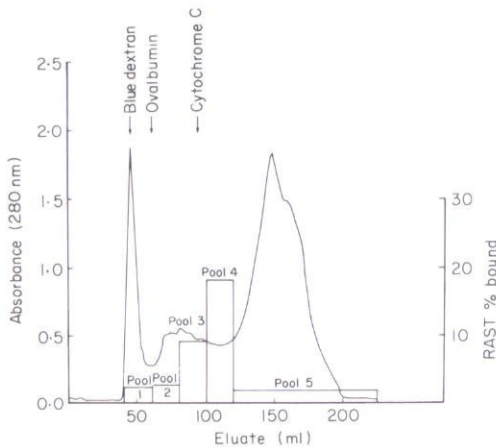


Fig. 1. Gel filtration of *Coprinus quadrifidus* spore extract on a Sephadex G-75 column. One to three grams of extract were applied to the column and 10 ml column fractions collected drop-wise. Fractions were analysed for ultraviolet absorption (—), pooled, and analysed for allergen activity by RAST (□).

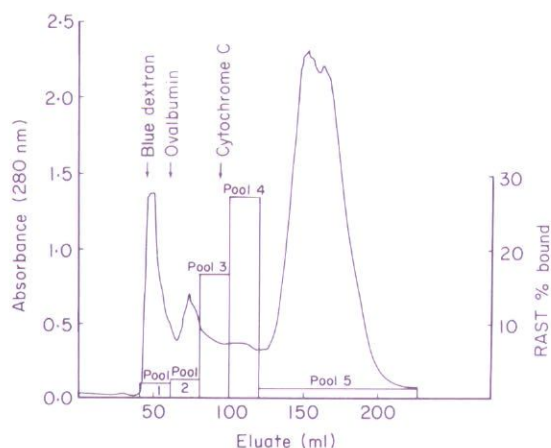


Fig. 2. Fractionation of *Coprinus quadrifidus* cap extract on a Sephadex G-75 column. The column fractionation was performed as described in Fig. 1. (—) Absorbance, (□) RAST.

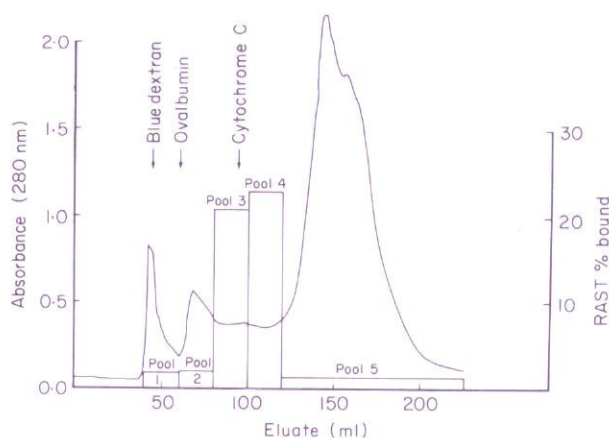


Fig. 3. Fractionation of *Coprinus quadrifidus* stalk extract on a Sephadex G-75 column. The column fractionation was performed as described in Fig. 1. (—) Absorbance, (□) RAST.

spore, cap, and stalk extracts. As summarized in Table 1, eleven subjects were skin-test positive to at least one of the *Coprinus* extracts, as well as two or more of the common inhalants. Thirteen subjects were skin-test negative to the *Coprinus* extracts but were positive to at least two of the common inhalant allergens. Four subjects were negative to all allergens tested. Of the eleven subjects skin-test positive to *Coprinus*, five demonstrated RAST ratios greater than 2.5% (Table 1). All seventeen subjects that were negative by skin test to *Coprinus* were negative by RAST as well.

Since there appeared to be an association of RAST activity of spore with stalk, this was further analysed by linear regression analysis (Fig. 4). Spore RAST activity demonstrated a highly significant correlation with stalk RAST. No RAST activity was seen in patients who were skin-test negative to the *Coprinus* extracts.

Column fractions with the maximal RAST activity (pools 3 and 4) of spore, cap, and stalk extracts were used to inhibit spore discs coupled with spore extract (pools 3

Table 1. Skin-test and RAST reactivity of individuals to *Coprinus quadrifidus* spore and stalk extracts

Patient	Skin test*			RAST % bound	
	Spore	Cap	Stalk	Spore	Stalk
M.H.	10.0	1.0	1.0	3.33	10.14
R.P.	10.0	1.0	1.0	1.48	1.16
W.D.	1.0	1.0	1.0	6.50	7.63
K.R.	1.0	0.1	0.1	5.29	4.16
Jo.C.	0.01	0.01	0.01	1.81	1.18
A.O.	1.0	1.0	1.0	11.39	11.95
J.L.	1.0	1.0	1.0	0.98	0.70
H.C.	> 10.0	10.0	> 10.0	2.80	2.12
D.W.	> 10.0	> 10.0	1.0	1.16	1.23
Ja.C.	> 10.0	> 10.0	10.0	1.23	1.35
P.D.	> 10.0	> 10.0	10.0	2.03	1.65

* Lowest concentration in mg/ml yielding a mean weal diameter > 2 mm.

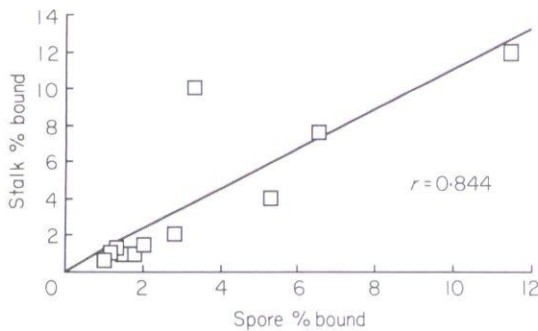


Fig. 4. Comparison of RAST activity to *Coprinus* stalk or spore extracts of sera from *Coprinus* skin-test positive individuals by linear regression analysis ($r = 0.844$; $P < 0.001$).

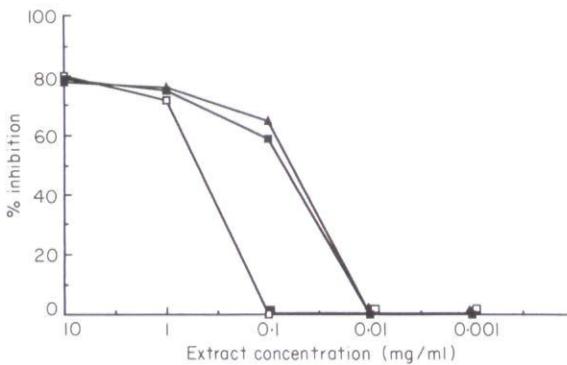


Fig. 5. Inhibition of *Coprinus quadrifidus* spore RAST with spore (□), cap (■), and stalk (▲) extracts. Increasing concentrations (0.001–10 mg/ml) of each extract were tested.

and 4). As seen in Fig. 5, cap and stalk extracts at concentrations of 10, 1, and 0.1 mg/ml gave RAST inhibition values comparable to, or exceeding, that of spore extract at the same concentration.

Discussion

Past and recent evidence strongly suggests that basidiospores may be important fungal aeroallergens in different parts of the world [3,4,6,7]. In New Orleans, 68% of asthmatic subjects had positive skin-prick tests to basidiomycete antigens [17]. Study of this group of fungi has been difficult because of the large amount of pure spores that are needed to prepare sufficient amounts of material for testing. In this study, crude extracts of spores, caps, and stalks were made and fractionated by gel filtration on a Sephadex G-75 column. Allergenic activity, as determined by RAST, was found in fractions with a molecular weight of 10.5–25 kD in all three extracts. Extracts of cap and stalk, therefore, contained allergens that were similar in size to those in spore extract. Furthermore, RAST inhibition analysis demonstrated that cap and stalk allergens share determinants with spore allergens.

The partially purified extracts obtained from gel filtration were used for skin-prick testing and RAST analyses. The results show that patient reactivity to cap or stalk extract was similar to spore extract. In these studies, stalk extract appeared to be the most potent reagent.

These results are encouraging since they suggest sources of allergens other than spores, whose collection and processing is highly laborious. Larger quantities of *Coprinus* extract will be available if the entire mushroom may be extracted. However, further allergenic analysis of spore, cap, and stalk extracts, such as crossed immunoelectrophoresis, crossed radio-immunoelectrophoresis and 'Western' blotting, is necessary to establish firmly the acceptability of these alternate sources of spore allergens in clinical studies.

It is of interest that extracts of *Coprinus* cap and stalk contain allergens in amounts approximately equal to, or greater than, those present in spore extracts. Although cap extracts are contaminated with residual spores, it is impossible to account for all of the allergenic activity present in cap extracts by spores. Furthermore, it was demonstrated that the stalk, which does not have basidia and, therefore, minimal spore contamination, was highly active in RAST and RAST inhibition studies. These results suggest that mycelial components present in the stalk and cap share allergenic epitopes with those present in spores. The identity and function of these components remains unknown. Assuming that spore inhalation is the mode of allergen delivery to the bronchi, then the allergens are probably surface components easily eluted in the lung, stimulating allergic responses.

It is curious to note that *Coprinus* spore, cap, and stalk allergen stability was more variable than other basidiomycete extracts. It has been our experience that the addition of moisture, however slight, will increase the degradation process and result in reduced activity of the *Coprinus* extract. With *Coprinus* this may be related to the autolytic process that occurs via hydrolytic enzymes during spore release [11]. Some autodigestive enzymes may also destroy allergenic activity and thus render crude allergen extracts high unstable. Fractionation of crude extract by gel filtration immediately after lyophilization yields partially purified fractions that are relatively stable in comparison to crude extracts. One might speculate that this is due to the removal of autolytic enzymes.

In view of the numerous aeroallergens described in recent years, and the amount of time necessary to study these allergens, is it necessary to investigate additional aeroallergens such as basidiospores? Although evidence strongly suggests they cause respiratory allergies, the extent of this problem must be established in order to justify further testing. Elevated levels of basidiospores have been demonstrated by different investigators. We plan to analyse further skin-test reactivity to the same basidiospore extracts in seven major centres throughout the United States and Europe in order to assess the importance of basidiospore skin-test reactivity. Furthermore, we have begun challenge studies and have demonstrated significant changes in lung function (FEV₁) of skin- and RAST-test positive patients upon exposure to several basidiospore allergen extracts, including *Coprinus* [10]. These results conclusively establish that basidiospores cause respiratory allergies and warrant further study.

Acknowledgments

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