

Identification and analysis of basidiospore allergens from puffballs

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Spore extracts from four common puffballs (Calvatia cyathiformis, Geaster saccatum, Pisolithus tinctorius, and Scleroderma areolatum) were fractionated by Sephadex G-75 gel filtration. Pooled fractions, analyzed by skin test or direct RAST, contained allergenic components over a molecular weight range of 10,500 to 20,000 daltons for all puffball spores studied except for S. areolatum (>70,000 daltons). The Sephadex G-75 allergenic pool of C. cyathiformis was further fractionated by hydrophobic interaction chromatography. Fractions were successively eluted with 4 and 1 mol/L buffered saline solutions and water. The 1 mol/L fraction contained maximal allergenic activity as determined by RAST-inhibition studies. At least 15 Coomassie blue staining bands were identified after isoelectrofocusing, of which 7 were shown by immunoprinting to bind IgE from a serum pool of five patients with allergy. Our results demonstrate that a combination of gel filtration, hydrophobic interaction chromatography, RAST-inhibition, and immunoprinting studies can be used to fractionate, characterize, and standardize spore allergen extracts of puffballs for diagnostic and therapeutic purpose. (J ALLERGY CLIN IMMUNOL 1988;82:787-95.)

Basidiomycetes are morphologically complex fungi. The approximately 25,000 species of basidiomycetes include mushrooms, puffballs, rusts, smuts, and bracket fungi. High atmospheric concentrations of basidiospores have been found in many parts of the world.^{1,2} Earlier studies showed a correlation between elevated basidiospore levels in the environment and increased seasonal incidence of hospital admissions for acute asthma.³ More recently, positive skin and RAST reactions to basidiospore extracts have been demonstrated in patients with respiratory allergy.⁴⁻¹⁰ These reactions correlate with a questionnaire-derived history of respiratory symptoms.^{4, 5, 9}

Calvatia cyathiformis, Pisolithus tinctorius, Sclero-

Abbreviations used

HIC: Hydrophobic interaction chromatography
PBS: Phosphate-buffered saline
IEF: Isoelectric focusing
EG: Ethylene glycol
UV: Ultraviolet
ID₅₀: Amount that inhibits 50% RAST

derma areolatum (S. lycoperdoides), and Geaster saccatum are four puffballs of the gasteromycete group of basidiomycetes and are widely distributed in Europe and North America. Characteristic of puffballs is that the spore-bearing portion, or gleba, occupies the interior of the fruiting body and matures into a dry and powdery spore mass. The gleba, which nearly fills the entire fruiting body, is estimated to contain billions of spores.¹¹ Previous studies have shown that spores from these puffballs are significant aeroallergens in New Orleans, as demonstrated by positive skin test reactions and serum IgE antibody responses in patients with respiratory allergies.^{5, 10} The prevalence of skin test reactivity ranged from 7% with *P. tinctorius* to 17% with *Scleroderma* sp.

The aim of this study was to analyze allergenic components present in basidiospores from these four common puffballs.

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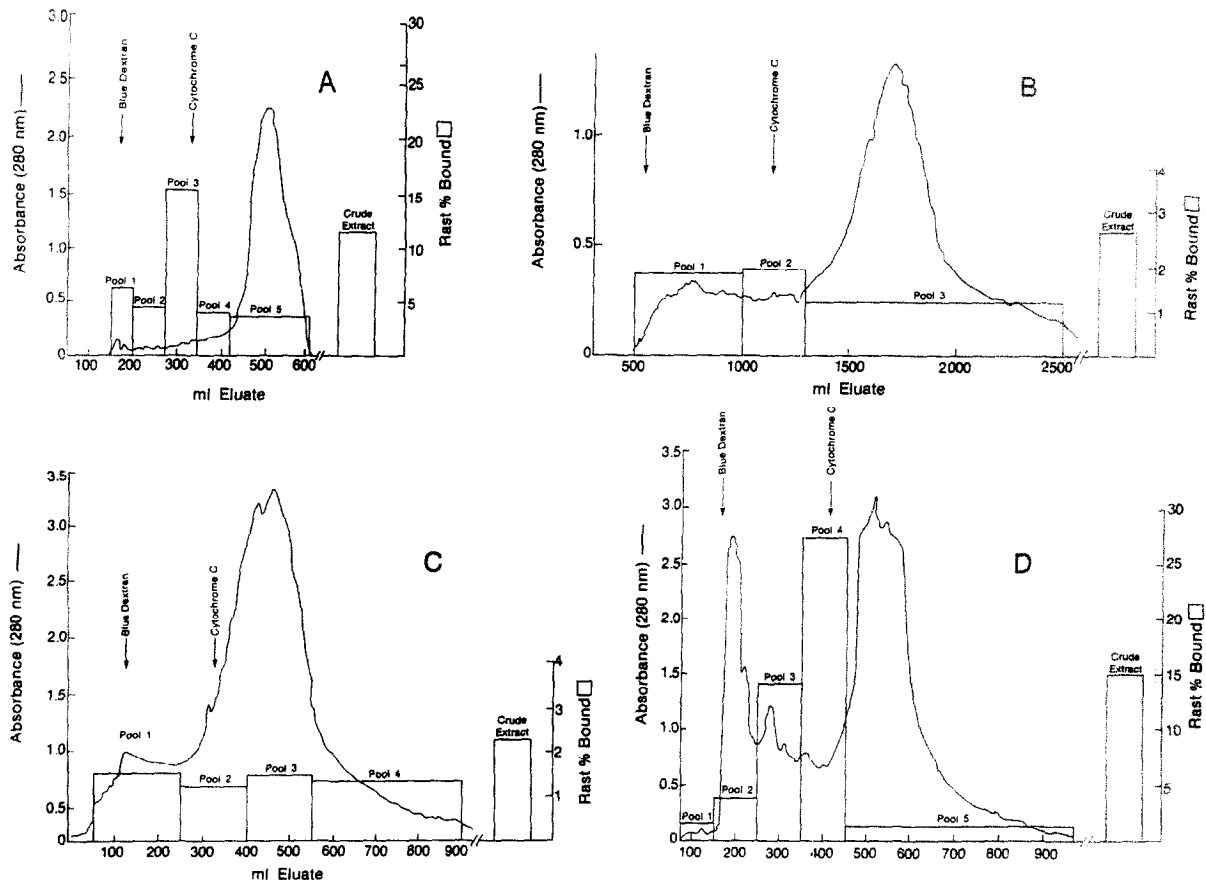


FIG. 1. Absorption spectra of *C. cyathiformis* (A), *P. tinctorius* (B), *S. areolatum* (C), and *G. saccatum* (D) after gel filtration on Sephadex G-75. Four to five hundred milligrams of extract was applied to each column and 5 ml column fractions were collected dropwise. Fractions were monitored for UV absorbance (280 nm) and peaks were pooled as indicated and analyzed for allergenic activity by RAST. Histogram bars indicate direct RAST values (percent label bound) of pooled fractions and crude spore extracts.

MATERIAL AND METHODS

Basidiospore extracts

Basidiospores were obtained and extracts prepared from four different species of puffballs (*C. cyathiformis*, *G. saccatum*, *P. tinctorius*, and *S. areolatum*) growing in the Gulf South area of the United States as previously described.¹² When harvested, puffballs were dried at room temperature for 1 or 2 days, then cut in half. The interior spore mass was removed with a spatula. Light microscopic examination of this material indicated at least 80% pure spores, with the major contaminant being sterile hyphae (capillitium). *C. cyathiformis* spores were defatted with ethyl ether (1 gm spores/dl) before extraction. Spores were homogenized in 0.125 mol/L ammonium bicarbonate buffer at pH 8.1 (1 gm spores in 20 ml) for 2 minutes at 4000 rpm and cooled with escaping CO₂ gas. The homogenate was centrifuged (80,000 × *g*) and the supernatant then lyophilized and stored under desiccation.

Sera

Serum samples with relatively high RAST reactivity were obtained from seven atopic subjects with symptoms of rhi-

nitis and/or asthma. These patients had positive skin prick test reactions to spore extracts of *C. cyathiformis*, *G. saccatum*, *P. tinctorius*, and/or *S. areolatum*. Separate pools of sera were prepared with equal volume of the individual sera (for each puffball) from four to five individuals positive by RAST and/or skin testing to that species. A control serum pool was prepared from atopic patients with negative skin test and RAST results to *C. cyathiformis* spore extract.

Skin test

All skin testing was performed on the volar surfaces of the forearm. Prick skin test end point titration was performed with 10-fold increasing concentrations (0.1 to 10 mg/ml) of *C. cyathiformis* and *S. areolatum*. Intradermal skin test end point titration to *P. tinctorius* was also performed with 10-fold increasing dilutions (0.001 to 1 mg/ml). Diluent was used as a negative control and histamine diphosphate (1 and 0.1 mg/ml for prick test and intradermal test, respectively) was a positive control. Wheals were measured after 15 to 20 minutes. Any extract inducing a mean wheal diameter 2 mm greater by prick test or 5 mm greater by

TABLE I. Prick skin test of *Calvatia cyathiformis*

Patient	Fraction tested* (mg/ml yielding positive reaction)					
	Crude	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5
R. P.	10	>10	>10	1.00	>10	>10
M. H.	0.10	>10	>10	0.10	>10	>10
F. B.	0.10	>10	1.00	1.00	>10	>10
W. D.	1.00	>10	>10	0.10	>10	>10

*Spore extract end point titration. A positive reaction was a mean wheal diameter 2 mm greater than the negative control.

TABLE II. Intradermal skin test of *Pisolithus tinctorius*

Patient	Fraction tested* (mg/ml yielding positive reaction)			
	Crude	Pool 1	Pool 2	Pool 3
W. D.	0.10	0.01	1.00	>1.00
M. H.	1.00	1.00	0.10	>1.00
F. B.	0.10	1.00	0.10	>1.00
R. P.	0.10	>1.00	0.01	>1.00

*Spore extract end point titration. A positive reaction was a mean wheal diameter 5 mm greater than the negative control.

intradermal test than that of the negative control was considered positive.

Gel filtration

Gel filtration chromatographic analysis was performed with a Sephadex G-75 column (2.5 cm × 100 cm for *C. cyathiformis*, *S. areolatum*, and *G. saccatum* or 5 cm × 100 cm for *P. tinctorius*). All columns were equilibrated with 0.125 mol/L NH₄HCO₃ at pH 8.1 at 4° C and calibrated with blue dextran (2 × 10⁶ daltons), ovalbumin (44,000 daltons), and cytochrome C (12,400 daltons). Four to five hundred milligrams of crude spore extract was re-suspended in 5 to 10 ml of 0.125 mol/L NH₄HCO₃ at pH 8.1 and centrifuged at 80,000 × g for 5 minutes. The supernatant was applied to each column and eluted with NH₄HCO₃ buffer. Eluate was collected in 5 ml fractions and monitored for UV absorption at 280 nm. Fractions were pooled, lyophilized, and stored under desiccation. Before gel filtration on Sephadex G-75, *P. tinctorius* spore extract was first filtered through a Sephadex G-10 column to remove a low-molecular-weight dye that bound irreversibly to the gel beads.

HIC

HIC was performed with a phenyl-Sepharose CL-4B gel (Pharmacia, Piscataway, N.J.) on a 1 × 30 cm column (23.55 ml bed volume). The column was equilibrated with 0.05 mol/L Tris buffer at pH 7.4 (HIC buffer) containing 4 mol/L NaCl at room temperature. The lyophilized *C. cyathiformis* G-75 pool with allergenic activity was suspended in HIC buffer with 4 mol/L NaCl and applied

to the phenyl-Sepharose column. The eluate was collected in 2 ml fractions and monitored for UV absorbance at 280 nm. Elution was performed successively with 4 mol/L NaCl Tris buffer, 1 mol/L NaCl Tris buffer, ion-depleted water, and 20% (vol/vol) EG. Each elution step was continued until the optical density at 280 nm reached baseline levels. Fractions with peak absorbance were pooled and designated according to the NaCl molarity of the buffer: 4 and 1 mol/L HIC buffer, water, and EG. Fractions were dialyzed exhaustively against 0.05 mol/L NH₄HCO₃ and lyophilized.

RAST and RAST-inhibition studies

Coupling of basidiospore antigens to cyanogen bromide-activated paper disks for subsequent use in RAST and RAST-inhibition studies was carried out as described by Ceska and Lundkvist.¹³ Lyophilized extracts were suspended in 0.1 mol/L borate buffer (pH 8.0) at 10 mg/ml (*C. cyathiformis*) or 25 mg/ml (*S. areolatum*, *G. saccatum*, and *P. tinctorius*). RAST was performed as previously described.¹⁴ One hundred microliters of the sera pool from skin test-positive individuals was incubated overnight at room temperature with antigen-coupled disks and washed three times with physiologic saline solution to remove unreacted serum. One hundred microliters of ¹²⁵I-labeled equine antihuman IgE (Kallestad, Austin, Texas) (diluted with saline solution to contain approximately 25,000 cpm) were added per disk and incubated overnight at room temperature. Unreacted isotope was removed with three saline solution washes and the activity of each disk was determined in a gamma counter (Gamma 5500, Beckman Scientific,

TABLE III. Prick skin test of *Scleroderma areolatum*

Patient	Fraction tested* (mg/ml yielding positive reaction)				
	Crude	Pool 1	Pool 2	Pool 3	Pool 4
A. O.	0.1	0.1	10.0	>10.0	>10.0
P. D.	0.1	0.1	1.0	>10.0	>10.0
M. H.	10.0	0.1	10.0	>10.0	>10.0
F. B.	0.1	0.1	1.0	>10.0	>10.0

*Spore extract end point titration. A positive reaction was a mean wheal diameter 2 mm greater than the negative control.

Palo Alto, Calif.). All tests were performed in duplicate and results were expressed as percent of total counts per minute added that bound to the disk.

RAST-inhibition studies were performed as described previously.¹⁵ Briefly, 50 μ l containing 10-fold increasing concentrations (0.001 to 10 mg/ml) of HIC fraction pools in PBS at pH 7.2 and 50 μ l of the RAST-positive sera pool were simultaneously added to disks coupled with the G-75 RAST active fraction pool of *C. cyathiformis* at 10 mg/ml. All tests were performed in duplicate, means were determined, and results were expressed as percent inhibition of the control RAST (inhibited with PBS).

IEF and immunoprinting

Thin-layer polyacrylamide gel (5% T/3% C) IEF was performed across the pH range 3.5 to 9.5 following the manufacturer's recommendations for power limits and total time (LKB PAG plates, Bromma, Sweden). Forty microliters of each HIC fraction of *C. cyathiformis* (25 mg/ml) was applied to the PAG plate on duplicate halves of the gel. A lane with pI markers was included on the gel half to be stained with Coomassie blue.

On completion of IEF, half of the gel was removed, fixed in a solution of 11.5% trichloroacetic acid and 3.5% sulfosalicylic acid, and stained in Coomassie brilliant blue. The remaining half of the gel containing focused proteins was blotted by passive transfer to 0.2 μ m pore size nitrocellulose membrane that had been activated with cyanogen bromide.¹⁶ After blotting, the nitrocellulose membrane was incubated for 1 hour in 0.04 mol/L phosphate buffer at pH 7.5 with 1.0% bovine serum albumin and 0.4% Tween 20 (blocking buffer) to block unreacted sites, and then washed with phosphate buffer containing 1% Tween 20.

The portion of the nitrocellulose membrane corresponding to each lane of the gel was divided lengthwise into two strips. One of each pair of strips was incubated with a RAST-positive serum pool. The remaining strip of each pair was incubated with a serum pool from five atopic individuals with negative RAST and skin tests to *C. cyathiformis*. The sera pools were adjusted to similar concentrations of total IgE by dilution with blocking buffer. The nitrocellulose strips were then washed in phosphate buffer/Tween 20 and incubated overnight in ¹²⁵I-labeled equine antihuman IgE diluted with blocking buffer (250,000 cpm/strip). After

washing to remove unbound labeled anti-IgE, autoradiographic analysis was performed on x-ray film at -80° C for 48 hours.

RESULTS

Gel filtration chromatographic analysis of crude basidiospore extracts demonstrated UV-absorbing material of varying molecular weights. Spectrophotometric analyses detected only one major peak in the eluate of *C. cyathiformis* (Fig. 1, A), *P. tinctorius* (Fig. 1, B), and *S. areolatum* (Fig. 1, C). Two major and several minor peaks were demonstrated in the eluate of *G. saccatum* (Fig. 1, D). Based on the spectrophotometric patterns, fractions were pooled as shown in Fig. 1 and skin test and/or RAST activity was determined for each pool. Gel filtration of crude spore extracts enhanced RAST activity by approximately 25% and 83% for *C. cyathiformis* (Fig. 1, A) and *G. saccatum* (Fig. 1, D), respectively. However, no significant increase in RAST activity of *P. tinctorius* and *S. areolatum* extracts was obtained after gel filtration. The maximal RAST activity after gel filtration of *C. cyathiformis* (pool 3; Fig. 1, A) and *G. saccatum* (pool 4; Fig. 1, D) was associated with column eluate of relatively low UV absorbance. These fractions eluted in the molecular weight range of approximately 10,500 to 25,000 daltons.

Allergenic activity in column fractions was further characterized by determination of skin test activity. Skin test results indicated that the G-75 pool 3 of *C. cyathiformis* spores—the fraction with maximal RAST activity—also contained maximal skin test activity. In other words, this pool induced reactions at the greatest dilutions in all subjects tested (Table I). Although significant skin test results were obtained with crude spore extracts and several G-75 fractions of *P. tinctorius* (Table II) and *S. areolatum* (Table III), they contained little RAST activity even after gel filtration. Pool 2 of *P. tinctorius*, with a molecular weight between 7,500 to 20,000 daltons and low UV absorbance, induced a positive skin test result at the

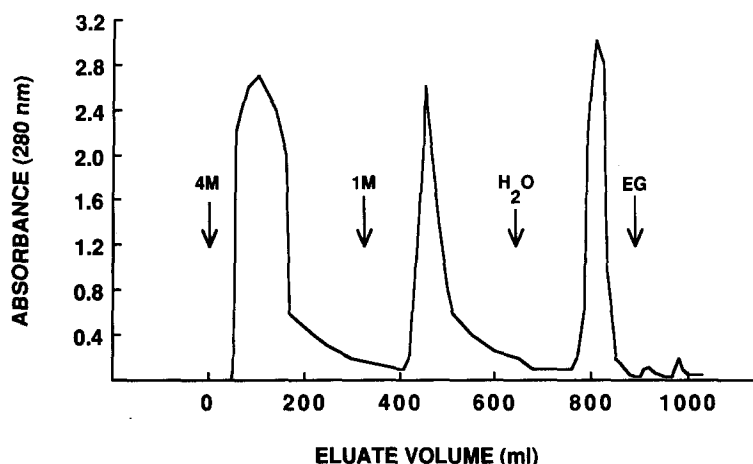


FIG. 2. Absorption spectra of HIC fractions of *C. cyathiformis* Sephadex G-75 pool 3. Elution was performed by stepwise desorption with 0.05 mol/L Tris at pH 7.4 with 4 mol/L NaCl (**4M**), 0.05 mol/L Tris at pH 7.4 with 1 mol/L NaCl (**1M**), water, and EG.

greatest dilutions in all subjects tested. Pool 1, of larger molecular weight, also induced positive skin tests in three of the four subjects tested (Table II). In contrast with other puffball spores studied, pool 1 of *S. areolatum*, which eluted in the void volume of the column (molecular weight >70,000 daltons), induced the highest skin test reactivity of all *S. areolatum* pools tested (Table III).

To fractionate *C. cyathiformis* allergens further, the Sephadex G-75 pool of *C. cyathiformis* containing the greatest skin test and RAST activity (pool 3) was applied to a phenyl-Sepharose CL 4B column (Fig. 2). Proteins with little hydrophobicity eluted in the 4 mol/L fraction, representing 54.8% of the material recovered. Subsequent desorption with 1 mol/L NaCl, H₂O, and EG yielded 29.7%, 11.1%, and 4.1% of the material recovered, respectively. The allergenic activities of starting material and HIC fractions were evaluated by RAST inhibition testing (Fig. 3). By graphic interpolation, the amount that inhibits 50% RAST (ID₅₀) was estimated to be 0.020 mg/ml for the G-75 allergenic fraction, 0.028 mg/ml for the 1 mol/L fraction, 0.17 mg/ml for the H₂O fraction, and 15 mg/ml for the 4 mol/L fraction (estimated by extending the inhibition curve). The total activity (ID₅₀) recovered represented 16.4% of the activity added to the column. Most (94%) of the allergenic activity recovered was in the 1 mol/L fraction (Table IV). No other proteins appeared to be retained in the column, because only trace amounts were eluted with EG (Fig. 2), none of which contained allergenic activity as detected by RAST-inhibition studies (Table IV).

C. cyathiformis HIC fractions were analyzed by IEF. Coomassie blue staining revealed >15 different protein bands in purified allergenic fractions. The

most intensely stained protein bands were observed in an acid pH range between 3.6 to 6.8. These acid bands were observed in crude extract, pool 2 of G-75 fractionation, and the 1 mol/L and H₂O fractions from HIC. A basic band (9.3 pI approximately) was present in crude extract and the G-75 fraction but not in HIC fractions (Fig. 4).

An immunoprinting technique was used to identify *C. cyathiformis* spore components in crude extract and purified fractions that bind specific IgE antibodies (Fig. 5). This technique revealed a total of approximately nine bands that bound IgE from a serum pool from skin test- and RAST-positive individuals. The most intense radiostaining was observed with bands located between pH 3.6 and 6.8. There were approximately six to eight bands in this range from crude extract, the G-75 fraction, and the 1 mol/L fraction. Weaker radiostaining intensity was observed with the H₂O fraction. Several bands with radiostaining of varying intensity were present in both the 1 mol/L and H₂O fractions, but one band (pI approximately pH 5.5) was present only in the H₂O fraction. A basic band (pI approximately pH 9.3) that bound IgE antibody was observed in crude extract and G-75 allergenic fraction, but not always in the HIC allergenic fraction. Other fractions (4 mol/L and EG) showed minimal or no binding of IgE. Pooled serum from atopic individuals with a negative skin test to *C. cyathiformis* showed minimal or no detectable radiostaining (results not shown).

DISCUSSION

Basidiomycetes have been suspected of inducing respiratory allergy in exposed atopic individuals. Spores probably contain the most relevant allergens,

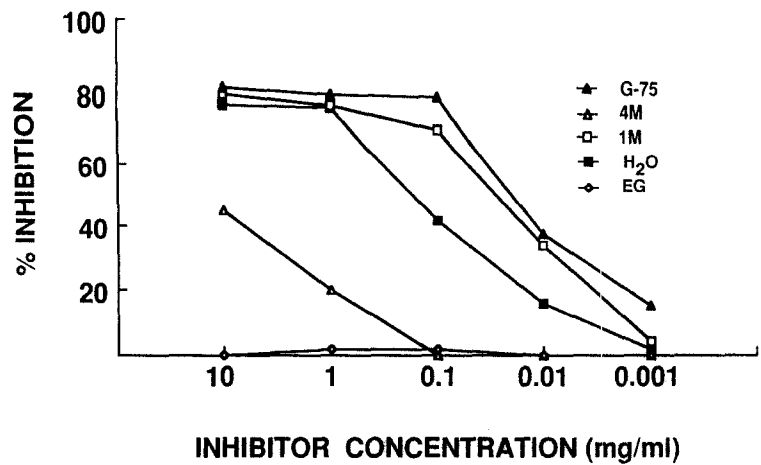


FIG. 3. Inhibition of *C. cyathiformis* Sephadex G-75 (G-75) pool 3 RAST by Sephadex G-75 pool 3 and HIC fractions. Increasing concentrations of *C. cyathiformis* fractions were tested for their ability to inhibit *C. cyathiformis* by RAST, 4M, 4 mol/L NaCl; 1M, 1 mol/L NaCl.

TABLE IV. RAST activity of HIC fractions of *Calvatia cyathiformis*

Substance tested	Specific activity in mg*	Total yield (mg)	Total activity† (ID ₅₀)	% Activity recovered
G-75 pool 3	0.020	970	48,500.0	100.00
4 mol/L	15.000‡	385	25.6	0.05
1 mol/L	0.028	209	7,464.0	15.39
H ₂ O	0.170	78	485.8	0.94
EG	—	30	—	—
Total		702	7925.4	16.38

*ID₅₀ in milligrams.

†Total activity is calculated by dividing total yield by ID₅₀.

‡Extrapolated.

because individuals are exposed primarily to spores rather than mycelia. Recently, the suitability of puffball spore extracts has been demonstrated in studies of basidiomycete-induced allergy.^{5, 8-10} The potential for puffballs to stimulate specific IgE antibodies in susceptible individuals may be greater than other basidiomycetes due to the large numbers of spores that puffballs release into the environmental spore load. In a study performed in New Orleans, spore extracts of *Scleroderma* sp., a puffball, showed the highest prevalence of skin reactivity (17%) among 15 basidiospores tested in adults with symptoms of respiratory allergy.⁵

The fractionation of crude spore extracts of *C. cyathiformis*, *P. tinctorius*, and *S. areolatum* by Sephadex G-75 resulted in similar UV absorbance patterns. One major peak of UV-absorbing material was present in the eluent of each spore extract. However, *G. saccatum* spore extract contained three major and several minor peaks of UV-absorbing material. Gel filtration

fraction pools, monitored by skin test and/or direct RAST, contained allergenic components over a molecular weight range of 10,500 to 20,000 daltons for all puffball spores studied except for *S. areolatum*. This molecular weight range is similar to that reported for many other allergens, including those from basidiospores of gilled mushrooms such as *Coprinus quadrididus*¹⁷ and *Pleurotus ostreatus*¹⁸ and also from deuteromycetes such as *Aspergillus*, *Alternaria*, and *Cladosporium*.^{19, 20}

Gel filtration of crude spore extracts resulted in enhanced RAST activity for *C. cyathiformis* and *G. saccatum*. This is possibly due to removal of inhibitory material or more likely removal of inert material present in the G-75 active fraction resulting in allergen enrichment. A good correlation between positive skin test and positive RAST was demonstrated with G-75 fractions of *C. cyathiformis* spore extract.

Previous studies have shown little or no IgE binding by RAST with crude spore extracts of *P. tinctorius*

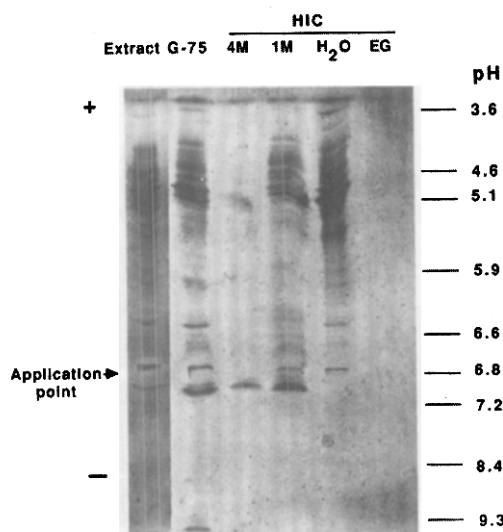


FIG. 4. IEF pattern of *C. cyathiformis* spore fractions after protein staining with Coomassie brilliant blue. Lane 1, Crude spore extract; lane 2, Sephadex G-75 (G-75) pool 3; lanes 3 to 6, fractions 4/mol/L (4M), 1 mol/L (1M), H₂O, and EG from HIC. Approximately 40 μ l of each sample at 25 mg/ml was applied to the gel.

and *S. areolatum*.^{10,12} Although it is possible that these extracts are too crude to provide effective RAST reagents, gel filtration fractionation failed to enhance RAST activity of these two basidiospore extracts. It is unlikely that our extraction protocol was not appropriate for allergens from *P. tinctorius* and *S. areolatum* because both *S. areolatum* and, to a lesser degree, *P. tinctorius* spore extracts have been active skin test reagents in atopic subjects.⁵ A lack of correlation was previously shown between positive skin test and RAST results with crude spore extracts of *P. tinctorius* and *Scleroderma* sp.¹⁰ Our results also showed a discrepancy between skin test and RAST results for these basidiospores, because G-75 fractions had skin test reactivity but no RAST activity. Why these basidiospore extracts were active by skin test but not by RAST remains to be determined.

The RAST reaction essentially binds protein antigens to cyanogen bromide-activated disks. It is possible that these basidiospore allergens have little or no free amino groups to bind to cyanogen bromide-activated hydroxyl groups on the disks. Although unlikely, these allergens could be nonproteinaceous molecules, such as polysaccharide, that cannot bind to RAST disks; thus, other methods must be used to insolubilize these allergens for in vitro measurement of specific IgE antibodies. Alternatively, if we are using optimal methods for IgE antibody detection, then it appears that *P. tinctorius* does not induce significant specific IgE responses. *P. tinctorius* gel fil-

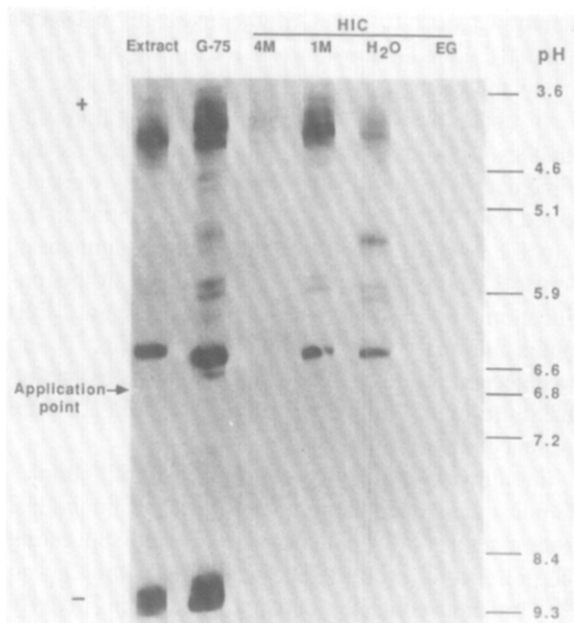


FIG. 5. Detection of IgE antibodies to *C. cyathiformis* proteins with a sera pool from patients allergic to *C. cyathiformis* by immunoblot autoradiographs of IEF samples as described in Fig. 4. Lane 1, Crude spore extract; lane 2, Sephadex G-75 (G-75) pool 3; lanes 3 to 6, fractions 4 mol/L (4M), 1 mol/L (1M), H₂O, and EG from HIC. Forty microliters of each sample at 25 mg/ml (1 mg/lane) was applied to the gel.

tration fractions showed less skin test reactivity than did fractions of other puffball species. This necessitated the use of an intradermal test, rather than a prick test, to obtain adequate skin reactivity. A possible explanation of this observation is that *P. tinctorius* spores are less allergenic than others, despite the tremendous numbers of spores that are released into the environment.¹¹

A phylogenetic correlation emerges from these results when one notes that two taxonomic orders are represented by the four puffball species included in this study. *Calvatia* and *Geaster* are in the order Lycoperdales, whereas *Scleroderma* and *Pisolithus* are in the Sclerodermatales. Gel filtration enhanced the allergenic activity in the Lycoperdales species, but not in the Sclerodermatales. Likewise, a good correlation between RAST and skin test activity was present in the species of the order Lycoperdales, but not in the Sclerodermatales. At present, however, the available information is not sufficient to assess the significance, if any, of this correlation.

HIC analysis²¹ has been successfully applied to the fractionation of *Aspergillus* somatic antigen.²² The hydrophobic properties of *C. cyathiformis* spore proteins provide an interesting approach to their frac-

tiation. Most (54.8%) of the material recovered from HIC eluted in the 4 mol/L fraction without binding to the column and contained no discernable activity either by RAST-inhibition or immunoprinting analysis. In contrast, the majority of allergens had some degree of hydrophobicity because they were detected by RAST inhibition and immunoprinting in the 1 mol/L and H₂O fractions. Thus, a large amount of non-IgE binding material can be removed in this purification step. The strong chaotropic agent (EG) eluted minimal UV-absorbing material (Fig. 2) and no detectable bands in the IEF gel (Fig. 4), indicating that little or no strongly hydrophobic proteins were present in our extract.

Immunoprinting of isoelectrofocussed HIC samples demonstrated a number of components that bound IgE from a sera pool of patients with allergy, suggesting the presence of multiple allergens. The 1 mol/L and H₂O fractions contained maximal IgE binding activity as determined by immunoprinting, in agreement with RAST-inhibition data. At least 7 of 15 Coomassie blue staining bands were able to bind IgE from the serum of patients with allergy in the purified HIC allergenic fraction. This indicates a high degree of allergenic heterogeneity even after two fractionation steps, although some of these bands may be degradation products or isomers of the same allergen.²³

We have observed some variability in the presence of a band with basic pI detected by immunoprinting both in the G-75 and HIC allergenic fractions (Fig. 5). The variability could result from some variations observed during preparative gel filtration column chromatography. It is also possible that the basic band could simply be less stable than the other allergenic bands or could represent degradation products. Early results of studies to analyze the variability of that basic band under way suggest it is less stable than the other allergenic bands.

Previous reports of allergenic activity of basidiospores have been based on the use of crude extracts. The partially purified fractions obtained by gel filtration column chromatography generally enhanced skin and/or RAST activity for most puffball spores tested. *C. cyathiformis* was chosen to fractionate allergens further because gel filtration provided an effective method of purification and sufficient quantities of spores are available to prepare reliable extracts. The allergenicity of *C. cyathiformis* spore extracts (crude and G-75 fraction) has been clearly demonstrated by both in vivo⁵ as well as in vitro^{10, 12} tests. With the use of HIC, a significant degree of purification of *C. cyathiformis* allergens was achieved. However, it is clear that further purification steps are necessary to obtain individual allergens.

Based on the tremendous number of spores released from puffballs and their demonstrated aeroallergenicity by skin test and RAST, further analysis of crude extracts is needed. Additional fractionation will be useful to provide better allergenic extracts for diagnostic purposes and even possible material for immunotherapy treatment in patients with spore-induced respiratory allergies. Experiments are currently in progress in our laboratory to isolate puffball spore allergens and to analyze their physicochemical properties.

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Antibody responses in watermelon sensitivity

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*Watermelon-specific IgE (WM-IgE) appears to mediate the oropharyngeal symptoms after watermelon ingestion. However, less than one fourth of watermelon-sensitive individuals (i.e., those with detectable WM-IgE) develop such symptoms after watermelon ingestion. In an attempt to separate symptomatic from asymptomatic persons, we measured watermelon-specific-IgE and IgG₄ concentrations in the sera of 29 watermelon-sensitive patients, 6 of whom were symptomatic. Although the mean WM-IgE level of the symptomatic group exceeded that of the asymptomatic group (p = 0.04), individual WM-IgE values failed to distinguish symptomatic from asymptomatic persons. No differences in WM-IgG₄ levels between the two groups could be found. Watermelon extract proteins were separated by isoelectric focusing and blotted to nitrocellulose membranes. The membranes were reacted with the individual sera of 14 watermelon-sensitive patients and then stained for IgE, IgG₁, and IgG₄ binding. Great variations in IgE, IgG₁, and IgG₄ binding patterns were found, but no subtype restrictions were discovered. Although the pattern of IgE-stained bands in the absence of corresponding IgG₁-stained bands was seen more frequently in symptomatic individuals, immunoblot patterns failed to separate symptomatic from asymptomatic persons. We conclude that the pattern of IgE, IgG₁, and IgG₄ responses to separated watermelon allergens was not predictive of clinical symptoms in a group of watermelon-sensitive patients. (*J ALLERGY CLIN IMMUNOL* 1988;82:795-800.)*

Practicing allergists commonly see individuals who describe the development of itching and/or swelling of the lips, tongue, or throat shortly after food ingestion. We have previously reported an association between oropharyngeal symptoms after the ingestion of

Abbreviations used

WM-IgE:	Watermelon-specific IgE
WM-IgG ₁ :	Watermelon-specific IgG, subclass 1
WM-IgG ₄ :	Watermelon-specific IgG, subclass 4
RW-IgE:	Ragweed-specific IgE
PBS:	0.05 mol/L phosphate and 0.1 mol/L NaCl at pH 7.5 with sodium azide 0.1%
FCS-PBS:	Fetal calf serum diluted with 0.05 mol/L phosphate and 0.1 mol/L NaCl at pH 7.5 with sodium azide 0.1%
BSA-PBS:	Bovine serum albumin diluted with 0.05 mol/L phosphate and 0.1 mol/L NaCl at pH 7.5 without azide

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