

Arthur Helbling
W. Elliott Horner
Samuel B. Lehrer

Section of Allergy and Clinical
Immunology, Department of Medicine,
Tulane University School of Medicine,
New Orleans, La., USA

Identification of *Psilocybe cubensis* Spore Allergens by Immunoprinting

Key Words

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Abstract

Previous studies established that *Psilocybe cubensis* contains potent allergens, and that a significant percentage of atopic subjects were sensitized to *P. cubensis* spores. The objective of this study was to identify *P. cubensis* spore allergens using isoelectric focusing (IEF) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoprinting. Coomassie blue staining of IEF gels detected approximately 20 bands between pI 3.6 and 9.3. Immunoprints obtained with 15 *P. cubensis* skin test- and RAST-positive sera revealed 13 IgE-binding bands; the most reactive were at pI 5.0 (80%), 5.6 (87%), 8.7 (80%) and 9.3 (100%). SDS-PAGE resolved 27 proteins ranging from about 13 to 112 kD. SDS-PAGE immunoprints conducted with 11 skin test- and RAST-positive sera demonstrated 18 IgE-binding bands; most sera reacted to 16 (82%), 35 (100%) and 76 kD (91%) allergens. Both electrophoretic procedures demonstrated a single allergen (at pI 9.3 and 35 kD) that reacted with all sera tested. This study corroborates the allergenic significance of *P. cubensis* spores and identifies the allergens of greatest importance.

Introduction

It has long been recognized that fungi induce respiratory allergies, but their importance as causative agents for bronchial asthma and rhinoconjunctivitis is still underestimated. Basidiomycetes are a major class of fungi but their allergenic significance is incompletely understood. They include the prevalent macrofungi: mushrooms, puffballs, bracket fungi, and also the rusts and smuts. In some parts of the world, basidiospores are a major component of the airborne spore load [1–5]. Early studies suggested that basidiomycete aeroallergens induce respiratory allergy in exposed subjects; however, detailed investigations were only recently pursued [6–8].

Subjects from many countries that have symptoms of

respiratory allergy also have significant skin test reactivity to basidiospore extracts [6, 7, 9–14]. The prevalence of skin test reactivity to different basidiospore species ranges from 16 to 91% in the literature; in general, results indicate that basidiospore sensitivity is similar to or exceeds the skin test prevalence of Fungi Imperfecti [9, 11–14].

Psilocybe cubensis (Earle) Sing. is popularly known as the 'magic mushroom', and belongs to the basidiomycete family Strophariaceae (Agaricales). This gilled mushroom is a prolific spore producer and is common in warm, humid areas in the United States Gulf Coast region, Mexico, the Caribbean and south to Argentina [15]. Related species are common in lawns and pastures throughout much of the United States. Numerous studies, both in vivo [9, 14, 16, 17] and in vitro [18–20] demonstrated the allergenic impor-

tance of *P. cubensis*. *P. cubensis* spores consistently produced one of the most frequent positive skin test reactions of several basidiomycetes tested in subjects with respiratory allergic disease from North America and Europe [9, 10, 14, 17]. Bronchoprovocation challenge tests with four sensitized asthmatic subjects using *P. cubensis* spore extract also induced both immediate and dual reactions [16]. The objective of this study was to identify the allergens of *P. cubensis* spores and determine their relative importance.

Materials and Methods

P. cubensis Spore Extract

Wild *P. cubensis* caps were collected, and spore prints obtained by the Florida Mycology Institute, Pensacola, Fla. That is, caps were placed, gills down, on filter paper, and sealed beneath additional filter paper. The spore prints were harvested the next day by scraping the paper. Spore morphology and purity (> 95%) were verified by light microscopy.

P. cubensis spores were defatted with ethyl ether (1 g of spores in 150 ml) for 4 h, collected by filtration and air dried overnight. Extracts were prepared by homogenizing 1 g defatted spores in a Braun homogenizer with cooling (Braun Melsungen AG, Melsungen, FRG) in 25 ml of 0.125 mol/l ammonium bicarbonate buffer (pH 8.1) for 2 min. The homogenate was centrifuged (27,000 g) and the supernatant lyophilized and stored at room temperature under desiccation. Protein concentrations were measured by Coomassie plus assay (Pierce, Rockford, Ill.) following the manufacturer's instructions.

Patient Sera

Sera were selected from 16 atopic subjects who were demonstrated in a previous study to be skin test- and RAST-positive to *P. cubensis* spores [9]. Two panels containing 15 and 11 sera each were prepared for isoelectric focusing (IEF) immunoprinting and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoprinting, respectively. Since 4 sera were available in limited quantities, only 11 sera were analyzed by SDS-PAGE. One subject's serum, tested by SDS-PAGE, was not probed by IEF. The control serum pool was composed of equal volumes of serum from 5 atopic subjects with negative skin test and RAST to *P. cubensis* spores.

RAST and Total IgE

RAST was performed according to the method of Ceska and Lundkvist [21]. Cellulose disks, activated with cyanogen bromide (CNBr), were coated with antigen at 1 mg dry weight (32 µg protein) per disk. Antigen-coated disks were incubated overnight with 100 µl undiluted test serum. After washing with physiologic saline (0.9%), 100 µl ¹²⁵I-labeled anti-IgE antiserum (Kallestad, Chaska, Minn.) containing 15,000 cpm was added. After overnight incubation and washing with saline, bound ¹²⁵I was determined in a gamma counter (Gamma 5500, Beckman, Irvine, Calif.). The results were expressed as the percent of total added radioactivity that bound to the disk. Serum total IgE levels were measured with Kallestad QuantiCLONE kits, using ¹²⁵I-labeled anti-IgE.

Isoelectric Focusing

IEF was performed in thin-layer polyacrylamide gels (pH range 3.6 to 9.3) with voltage, current and power limited at 1,500 V, 50 mA and 30 W, respectively, for 90 min (PAGplates, Pharmacia LKB, Piscataway, N.J.). A standard mixture of proteins with isoelectric point (pI) values ranging from pH 3.6 to 9.3 (Sigma Chemical Co., St. Louis, Mo.) was used to monitor the pH gradient and to determine the pI of each extract's individual bands. Thirty-two micrograms of protein of *P. cubensis* spore extract per lane was applied to IEF gels. Before staining, portions of gels were fixed in a solution of 3.5% sulfosalicylic acid and 11.5% trichloroacetic acid for 1 h. After equilibration in destaining solution for 20 min, the fixed gels were stained for 20 min in 0.1% Coomassie brilliant blue R 250 preheated to 60 °C. Focused proteins were blotted by passive transfer to a nitrocellulose membrane (0.2 µm pore size) previously activated with CNBr [22].

SDS-PAGE

SDS-PAGE was performed with 10% T/2.7% C separation gels and 4% T/2.7% C stacking gels in an SE 600 electrophoresis chamber (Hoefer Scientific Instruments, San Francisco, Calif.) following Laemmli's [23] method. Samples were boiled for 5 min in treatment buffer containing 0.125 mol/l Tris-HCl (pH 6.8), 2% SDS, 10% glycerol and 5% 2-mercaptoethanol. One milligram dry weight (32 µg of protein) of *P. cubensis* spore extract was applied to each lane, and the gels were electrophoresed at 60 mA (constant current) until the bromophenol tracking dye was approximately 1 cm from the bottom of the gel. Proteins were transferred electrophoretically for 1 h at 200 mA to CNBr-activated nitrocellulose in 0.1 mol/l Tris-glycine transfer buffer (pH 10.4) containing 20% methanol. Detection of IgE-binding proteins were performed as described for IEF gels.

Immunoprinting

After transfer of focused proteins onto the nitrocellulose membrane, unreacted sites were quenched with phosphate buffer 0.04 mol/l (pH 7.5) containing Tween 20 (0.4%) and bovine serum albumin (1%). Blotted and quenched membranes were washed in PBS with 0.05% Tween 20 and cut into strips 5 mm wide. Strips were incubated overnight first with 1 ml of patient (diluted 1:8), or control (1:2) sera; washed and incubated overnight with 50,000 cpm of radio-labeled anti-IgE. Labeled proteins were detected by autoradiography at -70 °C for 1-5 days. Blots were incubated with sera from skin prick test-negative subjects to control for nonspecific binding. Since experimental sera had greater total IgE concentrations, the control sera were used at a 4 times higher concentration. No control sera revealed any IgE binding.

Results

RAST and Total IgE

RAST and total IgE values of the patient and control sera are presented in table 1. The mean RAST (percent bound) values for *P. cubensis* spores were 15.0 (7.0-33.0) and 1.4 (1.0-2.0) for the patient and the control sera, respectively. The mean total IgE values were 752 IU/ml (115-2,600) for the patients and 203 IU/ml (40-500) for the controls.

Table 1. Skin test, RAST to *P. cubensis* spore extract, and total IgE of the 21 study subjects

	Skin test	RAST % bound	Total IgE IU/ml
Patients			
1	+	13.5	2,600
2	+	11.7	1,650
3	+	15.0	355
4	+	15.0	400
5	+	33.0	690
6	+	21.0	680
7	+	7.4	115
8	+	17.0	1,250
9	+	13.0	150
10	+	22.0	310
11	+	9.0	860
12	+	7.0	395
13	+	19.0	720
14	+	16.0	420
15	+	8.7	290
16	+	11.7	1,150
Controls			
17	-	1.4	105
18	-	1.7	310
19	-	1.0	60
20	-	1.0	40
21	-	2.0	500

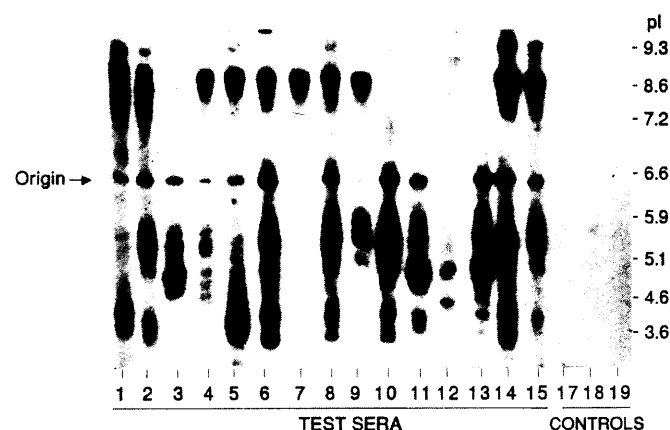


Fig. 1. IgE reactivity to *P. cubensis* spore allergens separated by IEF. Immunoprint pattern of crude *P. cubensis* spore extract with 15 positive and 3 negative test sera demonstrated 13 IgE-binding bands at pI 4.1–5.8, 8.7 and 9.3. The three most intense bands were at pI 5.0, 5.4 and 8.7.

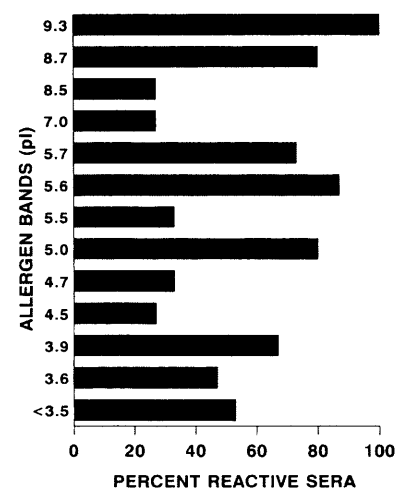


Fig. 2. Percent reactivity of patients' sera to *P. cubensis* spore allergens separated by IEF.

IEF/Immunoprint

Approximately 20 protein bands were detected in crude *P. cubensis* spore extract; the most intense were between pH 4.6 and 5.5 and at 9.3. Immunoprint patterns obtained from 15 *P. cubensis* RAST-positive sera demonstrated 13 IgE-reactive bands; their position corresponded with stained proteins (fig. 1). The number of allergens recognized by individual sera ranged from 4 (No. 7, 12) to 9 (No. 4). Eighty percent of the sera bound to at least 6 different IgE bands. The 2 most intense bands had pI 8.7 and 5.0. Double bands were recognized at pI 5.6 and 5.7 in 73% of test sera. Allergens with faint intensity were detected at pI 3.6, 4.5, 4.7 and 7.0. The greatest percentage of sera reacted with bands at pI 3.9 (67%), 5.0 (80%), 5.6 (87%), 5.7 (73%), 8.7 (80%) and 9.3 (100%; fig. 2). Five bands (< 3.5, 3.6, 3.9, 7.0 and 8.7) bound IgE, but apparently were not present in sufficient amounts to be detected by staining with Coomassie blue.

SDS-PAGE/Immunoprint

SDS-PAGE separated at least 35 proteins in crude *P. cubensis* spore extract between 13 and 112 kD. Immunoprints with 11 sera revealed 18 IgE-binding bands between 14 and > 100 kD (fig. 3). The most reactive bands were about 14 (73%), 16 (82%), 35 (100%), and 76 kD (91%; fig. 4). Individual serum recognized between 3 (No. 5) and 15 (No. 4) allergens. Eighty-two percent of the sera recognized at least 7 IgE allergens. All allergens were aligned with protein bands in the stained gels.

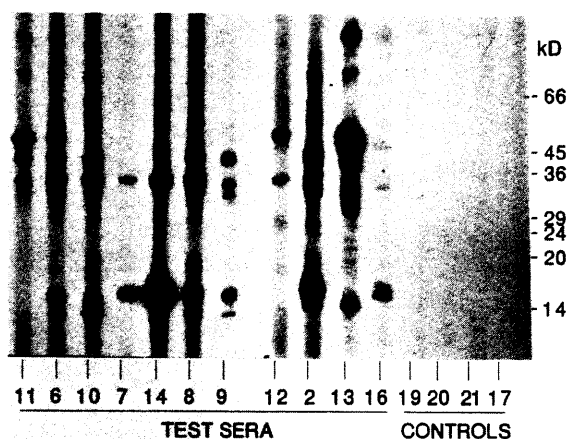


Fig. 3. IgE reactivity to *P. cubensis* spore extract resolved by SDS-PAGE. Immunoprint pattern of crude *P. cubensis* spore extract with 11 positive and 4 negative test sera demonstrated 18 IgE-binding bands from 13 to >100 kD. The three most intense bands were at 40, 35 and 16 kD.

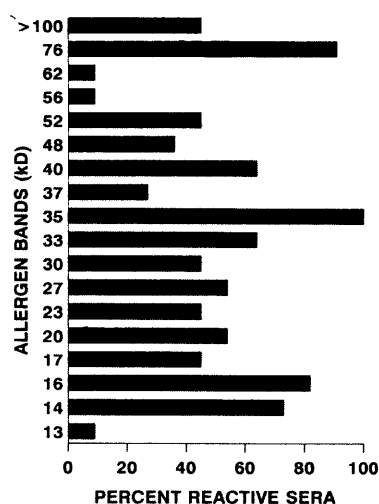


Fig. 4. Percent reactivity of patients' sera to *P. cubensis* allergens resolved by SDS-PAGE.

Discussion

Evidence indicates that *P. cubensis* spores are important basidiomycete aeroallergens [9, 16, 18–20]. The spore allergens also cross-react with allergens from certain other Basidiomycetes [19, 20]. However, *P. cubensis* spore allergens have yet to be characterized. This characterization is essential for diagnostic and possible therapeutic uses.

The present study demonstrates that crude *P. cubensis* spore extract is a complex mixture of proteins and allergens. The most reactive allergens, four detected by IEF and three by SDS-PAGE, bound IgE from more than 80% of the sera. By each electrophoresis method, one allergen, at 35 kD and pI 9.3, reacted with all sera tested. Such highly reactive allergens have not been reported from other Basidiomycetes investigated [24–30]. The most reactive basidiomycete allergen yet described, designated as *Cal c* Bd9.3 from *Calvatia cyathiformis*, reacted with only 68% of test sera. The number of allergens recognized varied among sera. However, sera with higher *P. cubensis* spore RAST values were more likely to demonstrate multiple IgE-binding bands in immunoprints than were sera with lower RAST values. Nonspecific IgE binding is unlikely, since negative control sera with similar total IgE levels showed no reactivity.

P. cubensis spore extracts are very potent compared to other basidiomycete allergens, as shown by in vitro [18–20] and in vivo [9, 14, 16] tests. *P. cubensis* generally gives higher RAST values than other species; furthermore, RAST inhibition showed that *P. cubensis* spores were the most potent inhibiting allergen among the Basidiomycetes investigated. Skin test surveys of atopic subjects from North America and Europe demonstrated that *P. cubensis* spore extract had the most frequent positive responses from all basidiospores tested and were comparable to *Alternaria* and *Cladosporium* of the Fungi Imperfecti [9, 10, 17]. This implies that *P. cubensis* spores may be useful as a model allergen for basidiospore allergy.

Although both IEF and SDS-PAGE are useful for analyzing crude *P. cubensis* spore extracts, SDS-PAGE provided better resolution for immunoprint and for protein staining. This is surprising since IEF is generally considered to better maintain the integrity of antigens and generally yields greater resolution. Thus some *P. cubensis* allergens may have identical pI values (isoallergens), but different molecular weights.

The pI 9.3 allergen is of particular interest since this band also occurs in and shares determinants with other Basidiomycetes [20, 24, 27]. Although the 9.3 band from *P. cubensis* (*Psi c* Bd9.3) had lower IgE-binding intensity than other *P. cubensis* allergens, the *Psi c* Bd9.3 did react with all sera tested. Unpublished studies indicate that the pI 9.3 band in *P. cubensis* has a molecular weight of less than 20 kD. This is similar to the *Cal c* Bd9.3, the pI 9.3 allergen from *P. cyathiformis* spores which has a molecular weight of 16 kD [25]. The 9.3 allergen cross-reacts by immunoprint inhibition among *C. cyathiformis*, *Pleurotus ostreatus*, *Coprinus quadrifidus* and *P. cubensis* [20]. Shared

epitopes of this allergen suggest evolutionary homology. However, convincing evidence of this homology requires further structural analysis of purified pI 9.3 allergen, such as amino acid sequence comparison and epitope comparison with monoclonal antibodies. Based on available characteristics, *P. cubensis* is a promising candidate for allergen analysis.

This study shows numerous proteins of crude *P. cubensis* spore extract that bind IgE from individual sera. Major allergens at pI 9.3 and 35 kD are candidates for isolation and might be useful agents to standardize *P. cubensis* allergen extracts for diagnostic and therapeutic uses. This study provides additional evidence that *P. cubensis* spores are major basidiomycete allergens.

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