

Stability Studies of *Calvatia cyathiformis* Basidiospore Allergens¹

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Abstract. *Calvatia cyathiformis* allergens in unfractionated extract (crude), and in extract sequentially fractionated by gel filtration (GF) and hydrophobic interaction chromatography (HIC) were tested for stability. *C. cyathiformis* allergen sources (crude, GF, HIC) were sampled immediately (0 h), or incubated at 4, 24 or 37°C and then sampled at 8, 24 or 96 h. Polyacrylamide gel-isoelectric focusing immunoprints revealed 3 allergen(s) groups, or bands (Bds) with respective pI of 3.6-4.6, 6.6 and 9.3. Only Bds 3.6-4.6 were stable at 37°C. At 24°C, Bds 3.6-4.6 persisted to 96 h, Bd 6.6 persisted 24 h, and Bd 9.3 waned in 8 h. At 4°C all 3 allergens in HIC were stable for 8 and 24 h; Bd 9.3 was reduced at 96 h. All allergen activity was labile to low pH conditions except for Bds 3.6-4.6. Proteinase K degraded Bd 9.3 more rapidly than Bd 6.6. Immunoprint patterns corresponded to the stained gels and were consistent among different sources. Bd 9.3 is very labile, but reactive with 63% of sera tested. Since 10-15% of *C. cyathiformis* reactors bind IgE only to Bd 9.3, this is notable variability, and significant for diagnosis and treatment.

Introduction

Basidiomycetes are a prevalent group of macrofungi which include mushrooms, bracket fungi and puffballs. The predominant mode of dispersal among these fungi is by the prolific production of airborne spores [1, 2]. It has long been known that the spores of basidiomycetes (basidiospores) are a major component of the airborne spore load in both urban and rural areas [3-8].

Recent studies indicate that 32% of patients with respiratory allergic disease were skin-test-reactive to at least 1 basidiospore extract - of a panel of 15 - thus establishing the clinical relevance of basidiomycete allergens [9]. Characterization of these allergens is central to a further understanding of their importance. We chose the puffball *Calvatia cyathiformis* (Bosc) Morg. as a model system for characterization of basidiomycete allergens.

A previous report from our laboratory established that crude and fractionated *C. cyathiformis* basidio-

spore extracts were capable of binding IgE from pooled sera of atopic individuals [10]. We also characterized the reactivity of these allergens with individual sera [11]. Some variability existed in these studies regarding allergen presence at different stages of fractionation, notably allergen *Cal c* Bd 9.3 [11]. We were unable to determine from those studies whether allergens were lost due to fractionation or lability.

The stability/lability of allergen extracts has received the attention of several workers [12-15]. Allergenic extracts of *Alternaria alternata* (= *A. tenuis*) are reported to be stable in lyophilized form for 12 months at all temperatures tested [12]. Stability of extracts in liquid was enhanced by the use of stabilizers and preservatives such as glycerol, human serum albumin and ϵ -aminocaproic acid [13-15]. These findings did not resolve our question though, since the study purposes were to quantitate the potency loss of extracts used in clinical settings and devise means of preventing such loss. These tests were also made with crude extracts and were concerned with total reactivity rather than tracking the loss of individual allergens in a mixture. Finally, *A. alternata*, the only fungal extract included, is only distantly related to the basidiomycetes and may have different properties.

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Thus, we initiated a study of the stability of individual allergens present in our extracts of *C. cyathiformis*. Specifically, we have evaluated the persistence of IgE-binding abilities under conditions of varying time and temperature, at different pH values and in the presence of proteinase K.

Materials and Methods

Spore Collection and Extraction

Basidiocarps (puffballs) were collected fresh in the Gulf South area and allowed to dry in the laboratory for 2–3 days. The spore mass was removed from the interior of the puffball with a spatula, screened through a kitchen strainer and stored in glass jars until used. Material thus obtained was about 85% spores, as determined by light microscopy; the balance of material was sterile hyphae that occurs among the spores in some puffballs (capillitium). Spores were defatted with ether, allowed to dry overnight, then homogenized in 0.125 M NH_4HCO_3 at pH 8.1 for 2 min in a Braun (Braun-Melsungen, Melsungen, FRG) homogenizer cooled with escaping liquid CO_2 . The homogenized suspension was centrifuged for 30 min at 80,000 g, and the supernatant lyophilized. Lyophilized crude extract was stored under desiccation at 24°C until used.

Chromatography

Chromatographic separations were performed as described previously [10]. Briefly, spore extract was fractionated by gel filtration and hydrophobic interaction chromatography (HIC). Gel filtration (GF) was performed in a 5 × 100 cm column with Sephadex G-75 (Pharmacia, Piscataway, N.J. USA) in 0.125 M NH_4HCO_3 at 4°C. Fractions of 10 ml were collected dropwise and ultraviolet absorbance at 280 nm was recorded. Fraction pools including peaks and interpeak areas were lyophilized and stored as above.

HIC was conducted with the radioallergosorbent test (RAST)-active G-75 pool, corresponding to 'pool 3' of Ibanez et al. [10]. Lyophilized material from this pool was resuspended in 0.50 M Tris buffer, pH 7.4, with 4 M NaCl (Tris/4 M) for application to the phenyl-Sepharose column. Elution with Tris/4 M continued until absorbance at 280 nm returned to baseline. Further stepwise elutions were made in a similar fashion with Tris/1 M NaCl, water and finally with 20% (v/v) ethylene glycol. HIC pools were dialyzed exhaustively against 0.05 M NH_4HCO_3 at 4°C and lyophilized.

Sample Incubations

C. cyathiformis allergen sources (crude, GF, HIC) were resuspended in 1% glycine, pH 6.5, at 20 mg/ml and sampled immediately (0 h). The remaining portion of each source was aliquoted and incubated at 4, 24 or 37°C. Incubations were sampled at 8, 24 and 96 h, snap-frozen and stored (less than 2 weeks) at -80°C until analyzed.

Lyophilized crude spore extract was resuspended separately in 0.05 M ammonium acetate buffer at pH 4, 7 and 9, and sampled immediately (0 h). The remainder of each solution was incubated at 24°C and sampled at 8, 24 and 96 h. Samples were snap-frozen and stored at -80°C until analyzed.

Proteinase K (Sigma, St. Louis, Mo., USA) was resuspended in calcium acetate buffer (pH 7.9) at either 4.4 U/ml in 5 mM buffer or 29 U/ml in 18 mM buffer and used at 1:6 (v/v) to digest crude extract resuspended (20 mg/ml) in 1% glycine. Extract was stored at 4°C until digested at 24°C. Digests were made with proteinase K at 4.4 U/ml for 18 h and at 29 U/ml for 16 h and for 1.5 h. Replicate extract dilutions were made with buffer only and incubated in parallel as negative controls throughout. Times of mixing sample with enzyme were staggered to allow simultaneous collection. Samples were analyzed immediately at the end of the incubations.

Isoelectric Focusing and Immunoprinting

Procedures were as previously described [16]. Briefly, protein samples were focused in thin-layer polyacrylamide 'Pagplate' gels (5%T/3%C; LKB, Piscataway, N.J., USA) in duplicate on adjacent portions of the same gel for immunoprinting and for staining and separated immediately upon completion of focusing. The portion of a gel to be stained was fixed in trichloroacetic acid/sulfosalicylic acid prior to staining in 0.1% Coomassie brilliant blue R-250.

Blots were made of unstained, focused proteins by passive transfer to 0.2-mm pore size nitrocellulose membranes that were previously activated with cyanogen bromide [17]. Unbound sites on the blots were then blocked and the membrane was overlaid with serum. The serum pool used was prepared by combining equal volumes of five RAST- and skin-test-positive sera. Bound IgE was detected with radiolabeled anti-IgE at approximately 100,000 cpm per strip. Three washes in phosphate-buffered saline (PBS) with 0.05% Tween 20 followed each step. Strips were rinsed in PBS after the last wash, air-dried and autoradiographs prepared by exposure to X-ray film at -80°C. Autoradiographs were scanned in a model GS 300 scanning densitometer (Hoeffer, San Francisco, Calif., USA) in transmission mode.

Carbohydrate Staining

A gel portion with focused crude extract was stained by the periodic acid-Schiff (PAS) reagent procedure.

Results

Time and Temperature

Proteins and allergens in crude spore extract had different levels of stability in these incubation conditions (fig. 1, 2). Longer time periods and higher temperatures were associated with decreased intensity and number of protein bands (Bds) stained with Coomassie blue (fig. 1). Likewise, allergenic activity, as indicated by autoradiography, decreased with time and temperature (fig. 2). Both *Calc* Bd6.6 and *Calc* Bd9.3 lost allergenic activity by 96 h at 24°C; at 37°C, activity was lost after 8 h. Bds3.6–4.6 were the most stable. These bands only diminished in activity at 37°C after 24 h and at 24°C at 96 h. Similar patterns occurred in the gels stained for protein with Coomassie blue.

Activity loss due to time is seen in the densitometer

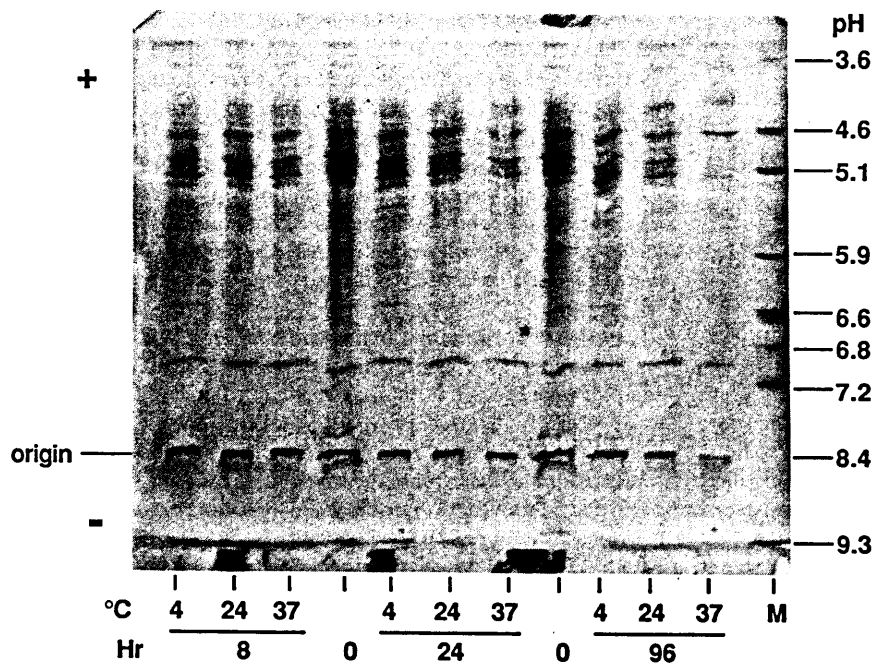


Fig. 1. Stability of crude spore extract components as evaluated by isoelectric focusing. Allergen solutions were sampled initially, then incubated at three temperatures and sampled again at three later time periods, as indicated beneath the lanes. Markers of known pI were included in lane M; origin denotes placement of application wick.

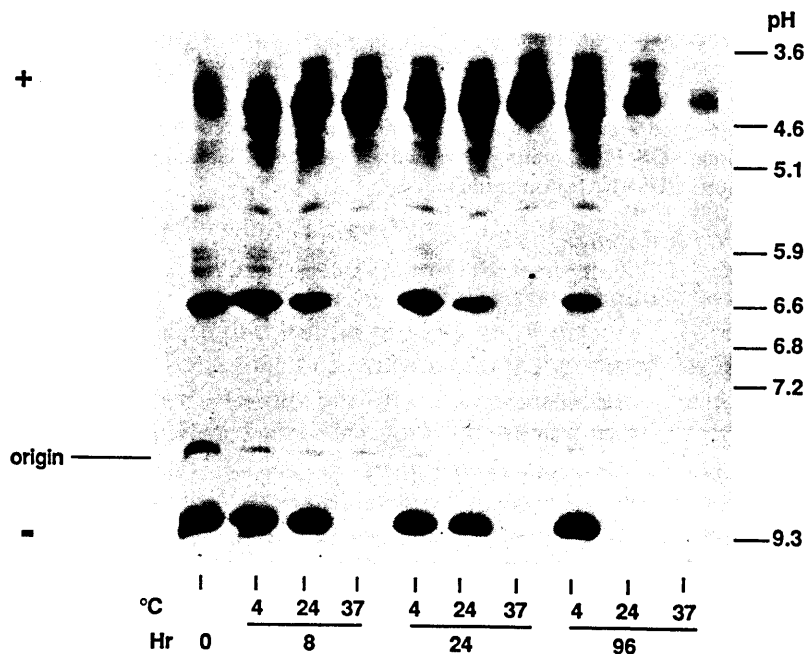


Fig. 2. Autoradiograph of allergens in crude spore extract detected by immunoprinting. Incubation conditions, position of origin and pH values are as in figure 1.

scans of the autoradiograph lanes for 24°C (fig. 3). In the scan of 8 h, the broad peak to the left corresponds to allergen activity at Bds 3.6–4.6, the center peak represents *Cal c* Bd 6.6 and *Cal c* Bd 9.3 is the peak to the right. Both *Cal c* Bd 6.6 and *Cal c* Bd 9.3 are diminished at 24 h and eliminated at 96 h. In figure 4 the effect of temperature is seen in the scans of lanes for 24 h. The peaks correspond to the major areas of al-

lergenic activity as seen in figure 3. At 24 h, *Cal c* Bd 6.6 and *Cal c* Bd 9.3 retained activity at 4°C, were somewhat reduced at 24°C and disappeared at 37°C.

Components in the GF fraction repeated this general pattern (data not presented). Lower amounts of *Cal c* Bd 9.3 were initially present in GF as indicated by the staining intensity in the gel and autoradiograph. *Cal c* Bd 9.3 showed the most rapid diminution

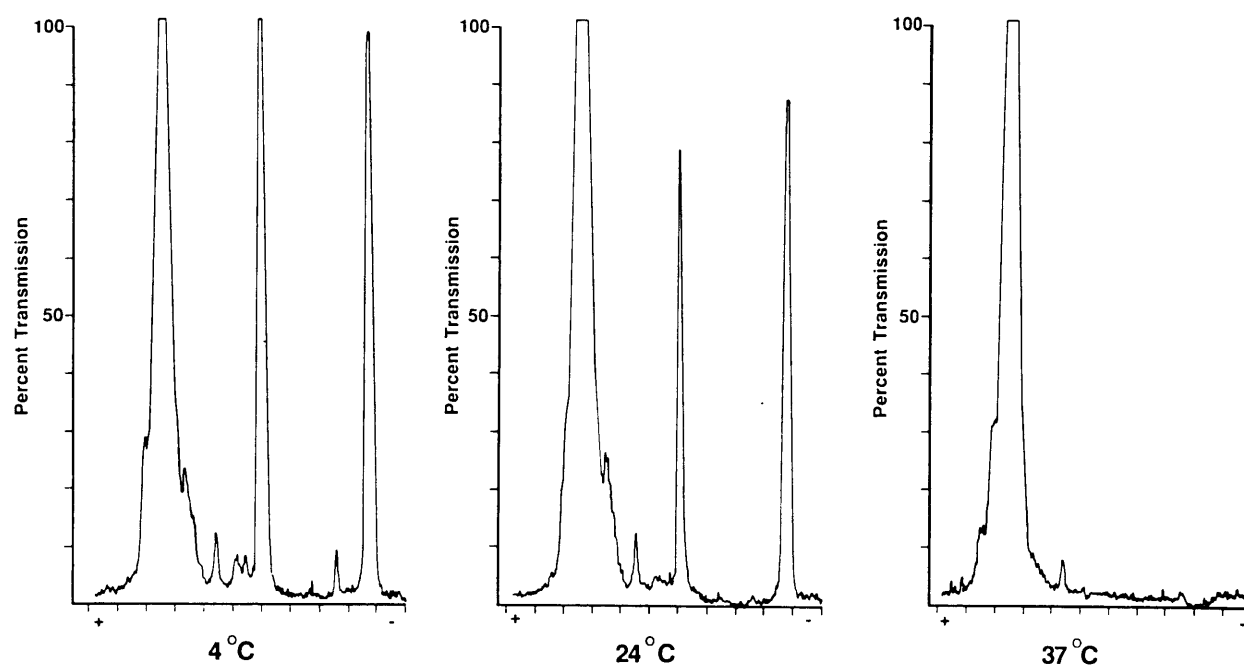


Fig. 3. Effect of temperature on stability of *C. cyathiformis* allergens. The tracings are densitometric scans of the autoradiograph (fig. 2) of lanes corresponding to samples incubated for 24 h at the indicated temperatures.

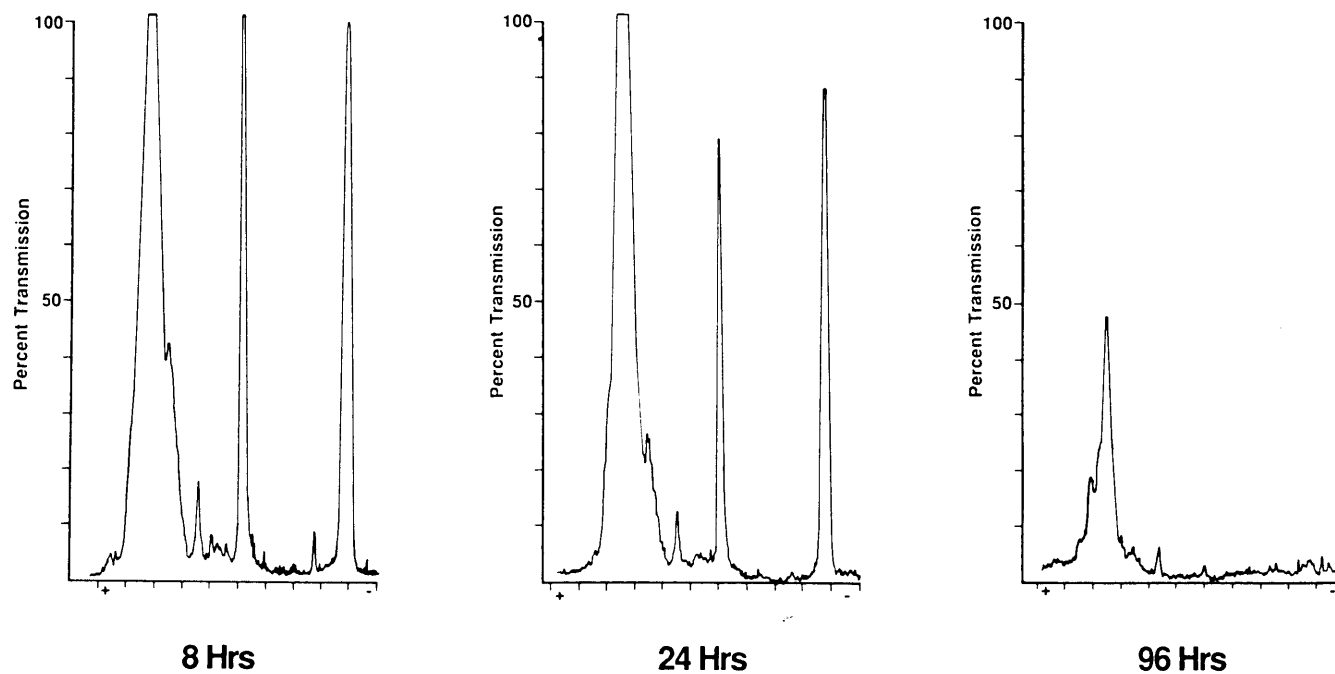


Fig. 4. Effect of time on stability of *C. cyathiformis* allergens. The tracings are densitometric scans of the autoradiograph (fig. 2) of lanes corresponding to samples incubated at 24°C at the indicated times.

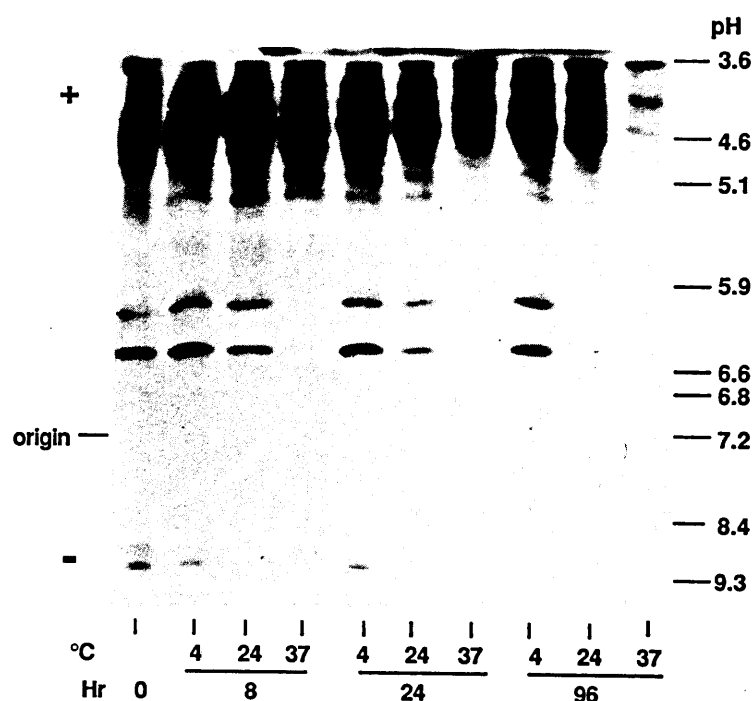


Fig. 5. Autoradiograph of allergens detected by immunoprinting in the HIC fraction of spore extract. Incubation conditions, and position of origin are as in figure 2. The pH values were transferred from the companion portion (stained) of the blotted gel.

of the allergens. Although *Cal c* Bd6.6 was enriched in the GF fraction relative to crude extract, this allergen diminished more quickly in the GF fraction than in the crude extract, not being detected at 24°C after 24 h. Again Bds3.6–4.6 were most stable. Neutral and low pH proteins were stained more intensely with Coomassie blue in the gel with samples from the GF fraction than in the gel with samples from crude extract.

Allergenic activity of Bds3.6–4.6 was intensified in the HIC fraction (fig. 5). As with the GF fraction, *Cal c* Bd9.3 was present in lower amounts in the HIC fraction than in crude extract and diminished in activity most rapidly of the allergens. *Cal c* Bd6.6 activity in the HIC fraction was similar to that in crude, although the amount of Coomassie blue staining was more intense.

The three predominant areas of allergenic activity correlated well with the earlier report of reactivity with individual sera [11]. These stability patterns of allergenic activity corresponded to the stained gels and were consistent among different sources. In general, the stability of the 3 important allergens were of decreasing order: Bds3.6–4.6 > *Cal c* Bd6.6 > *Cal c* Bd9.3.

Time and pH

The Coomassie-blue-stained gel indicated that many proteins, especially neutral and basic proteins, were degraded rapidly at pH 4 (fig. 6). Protein bands with $pI \leq 5.1$ were the most stable bands in the extract when incubated at pH 4. Their intensity was reduced more over time at pH 4 than at pH 7 or 9. Persistence of all bands was slightly better at pH 7 than at pH 9. Autoradiography indicated that all *C. cyathiformis* allergens except Bds3.6–4.6 were labile to pH 4 conditions (fig. 7). At pH 9, IgE-binding capability decreased over time with all allergens except Bds3.6–4.6. The greatest stability was seen at pH 7, where only moderate decreases in IgE-binding capabilities were seen at 96 h.

Proteinase K Digests

All three proteinase K digests reduced the IgE-binding activity in the immunoprint relative to the controls (data not presented). Increased time or enzyme concentration showed a greater reduction of activity. *Cal c* Bd9.3 lost activity in 1.5 h. IgE-binding activity of *Cal c* Bd6.6 was present at 1.5 h, but was greatly diminished at 16 h.

Fig. 6. Stability of crude spore extract components incubated at different pH values as indicated beneath each lane. Samples were evaluated by isoelectric focusing. Freshly resuspended extract was included as a control in lane C. Markers of known pI values were included in lane pI; origin denotes placement of application wick.

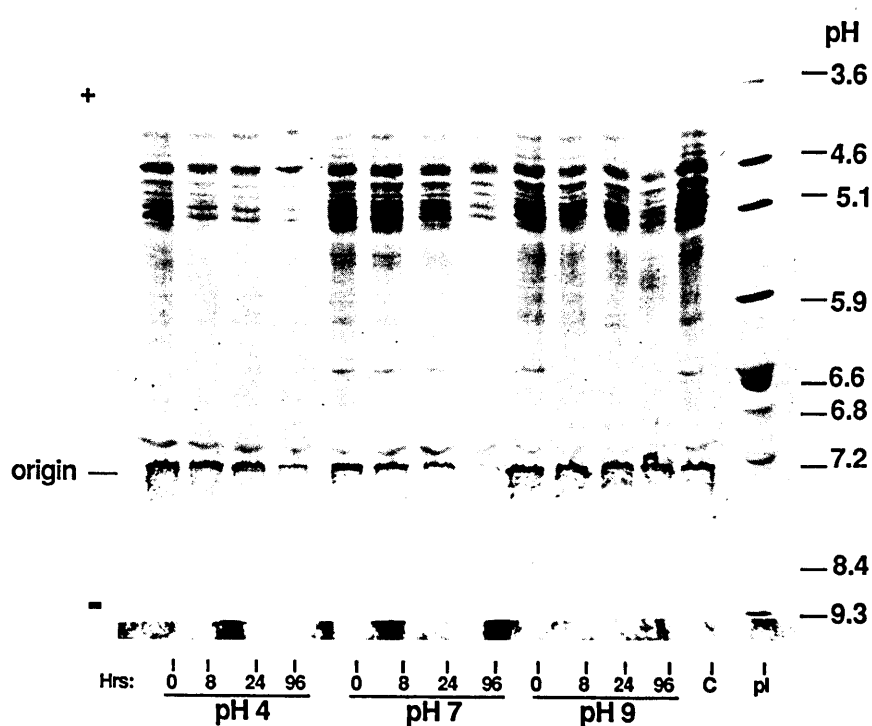


Fig. 7. Autoradiograph of allergens remaining in crude spore extract after the indicated incubation times and detected by immunoprinting. Position of origin and pH values were transferred from the gel in figure 6.



Carbohydrate and General Protein Staining

The PAS-stained gel portion revealed carbohydrate-containing bands in the gel region between pH 4.6 and 5.1 (fig. 8). This was the region of greatest concentration of Coomassie-blue-staining bands in the gel.

Discussion

Immunoprint analysis of allergens from *C. cyathiformis* showed that the basic allergens are less stable than the acidic allergens. In particular, *Cal c* Bd9.3 is a very labile allergen. The time and temperature study

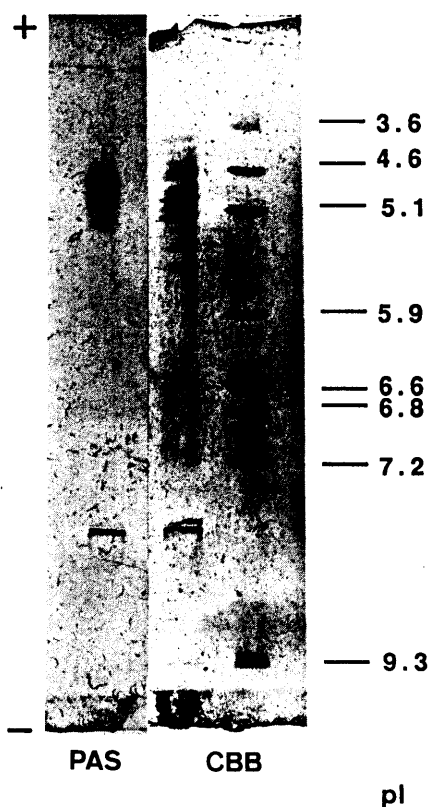


Fig. 8. General carbohydrate and protein staining of electrofocused crude extract. PAS reagent was used to stain carbohydrates and Coomassie brilliant blue R-250 (CBB) stained proteins.

demonstrated that *Calc* Bd9.3 lost activity more quickly than any of the other allergens. Both *Calc* Bd9.3 and *Calc* Bd6.6 lost activity during the same intervals when the crude extract was used, but in the HIC fraction *Calc* Bd6.6 activity persisted beyond the complete loss of *Calc* Bd9.3 activity. The pH 7 incubation using crude extract showed this as well.

Calc Bd9.3 lost activity rapidly in control incubations for the proteolytic digests. The activity of *Calc* Bd9.3, but not *Calc* Bd6.6, diminished in 16 h. This loss of activity may explain the low initial levels of *Calc* Bd9.3 in the time and temperature analysis of the HIC fraction. Since the HIC chromatography required 8 h and was conducted at room temperature, followed by a lengthy dialysis at 4°C to remove buffer salts, there was opportunity for loss of activity during the HIC procedure.

In both previous reports of *C. cyathiformis* allergens [10, 11], it was unclear whether *Calc* Bd9.3 was lost by fractionation or due to lability. The presence of *Calc* Bd9.3 in the HIC fraction at zero time indi-

cates that the allergen does persist through both fractionation steps, although reduced in amount. This may indicate that the yield of that band relative to other bands is less following GF fractionation, i.e. fractionation loss. The GF fractionation also provided an opportunity for loss by degradation, since these column runs could last up to 2–3 days, albeit at 4°C. These confounding factors preclude conclusions from these column runs regarding loss by fractionation versus loss by degradation.

The pH and the proteinase K sample incubations were conducted, however, with crude, i.e. unfractionated extract. *Calc* Bd9.3 was again the most labile band in both of these experiments. Thus, the present data indicate that the previously noted sporadic absence of *Calc* Bd9.3 may be explained by the lability of *Calc* Bd9.3, even though the possibility of fractionation loss cannot be excluded.

The stability of Bds3.6–4.6 and *Calc* Bd6.6 does not appear to be affected by purification. Activity loss patterns were similar in both crude and the HIC fraction for these bands. With *Calc* Bd9.3 the activity loss pattern seemed similar, but the overall intensity of *Calc* Bd9.3 was reduced in the HIC fraction, which complicates the comparison.

The effect of pH on allergen stability is indicated by the rapid loss at pH 4 of all IgE-binding activity except with the most acidic allergens. Even though protein bands persisted for some hours, the loss of allergen activity was essentially instantaneous. This suggests a physical denaturation of allergenic epitopes, followed by a slower degradation of the primary structure of the protein.

C. cyathiformis extracts, and possibly other basidiomycete extracts, should be maintained at neutral or slightly alkaline conditions when in solution, as during fractionation or dialysis. For the purposes of maintaining stability during purification efforts, the greatest emphasis should be placed on minimizing the amount of time that crude extracts are kept in solution.

Additional stability may be gained in clinical settings with a different buffer system or ionic strength, parameters that we did not consider. The addition of glycerol, human serum albumin and ϵ -aminocaproic acid have each been successfully used to stabilize allergen extracts in clinical settings and also may increase stability of mold extracts [12–15]. Extrapolation should be undertaken with care though, since *A. alternata* (= *A. tenuis*) was the only fungal species

included in any of these preservative/stabilizer studies.

Our results allow some speculation upon the chemical nature of these allergens. Loss of allergenic activity upon exposure to protease shows that the allergens – or at least the IgE-binding epitopes – of *C. cyathiformis* are proteinaceous. The PAS reagent staining indicates the presence of carbohydrate moieties – presumably glycoproteins – between pH 4.6 and 5.1, an area of low allergenic activity. Thus, we have no evidence for glycoprotein allergens although other fungal allergens are glycoproteins [18]. The PAS results should be interpreted with caution though, since carbohydrate moieties may have been present, but below the sensitivity limits of the PAS reaction.

Our earlier report [11] revealed that 10–15% of sera from *C. cyathiformis*-sensitive individuals reacted in vitro only to *Cal c* Bd9.3, a very labile allergen that can easily lose IgE-binding capacity. The clinical significance of these findings is that basidiospore-specific sensitivities may have been underestimated by previous surveys if lack of stability reduced the potency of the test extracts.

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