Preparative Isoelectric Focusing of Immunologically Reactive Components of *Aspergillus fumigatus* Mycelium

By ELAINE V. WILSON, SYLVIA W. DE MAGALDI AND VERONICA M. HEARN*

Mycological Reference Laboratory, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, UK

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A water-soluble mycelial extract of *Aspergillus fumigatus* has been fractionated by preparative isoelectric focusing using carrier ampholytes in a layer of granulated gel. The separated components were located by staining paper prints from the gel. Within a narrow pH range of 2.5 units, multiple protein bands were visualized with Coomassie Brilliant Blue G. Periodate-Schiff-positive material was generally associated with the major protein zones. When these fractions were eluted the total recovery, calculated on the basis of protein and carbohydrate analyses of the isolated fractions, varied between 20 and 60% of the applied material. Low recoveries were associated with low recoveries of protein; recoveries of carbohydrate were higher and less variable. The immunological activity and specificity of the eluted fractions were assessed in an enzyme-linked immunosorbent assay for the detection of IgG antibodies to *A. fumigatus*.

INTRODUCTION

Methods described for the fractionation of water-soluble components of *A. fumigatus* include ion-exchange and affinity chromatography, gel filtration and salt fractionation (Longbottom & Pepys, 1964; Kim et al., 1978; Hearn et al., 1980; Wilson & Hearn, 1983a). An inherent disadvantage of chromatographic separations is dilution of the relevant components while salt fractionation requires subsequent exhaustive dialysis to remove these ions. In the initial stages of protein and glycoprotein purification, preparative isoelectric focusing (IEF) has advantages because of its concentrating capacity and the high degree of purification achieved in a single step (Radola, 1973). Earlier experiments with analytical polyacrylamide gel electrophoresis of *A. fumigatus* extracts indicated the potential of electrophoretic methods as a separation technique (Hearn & Mackenzie, 1979). Recently, the separation of constituent molecules on the basis of their isoelectric points (pI) has been successfully applied to the culture filtrate antigens of *A. fumigatus* (Kurup et al., 1983). We have now fractionated the water-soluble extract of *A. fumigatus* mycelium in a one-step process, and can thus obtain components suitable as antigens for the serological diagnosis of aspergillosis, without prior concentration of sample or removal of ampholyte.

METHODS

Preparation of mycelial extract. *Aspergillus fumigatus* strain NCPF 2109 was grown in stirred culture in neutral glucose/peptone medium incubated at 30 °C for 3 d (Proctor, 1976). The mycelial mat was harvested, suspended in 0.05 M-NH₄HCO₃ at pH 8.0 and homogenized in a Dynomill cell disintegrator (Glen Creston, London, UK) at 4 °C (Hearn & Mackenzie, 1979). The supernatant obtained after centrifugation at 2000 g was reduced in volume

Abbreviations: ASS, ammonium sulphate supernatant; BF, carbohydrate-enriched; UBF, protein-enriched; WS, water-soluble; ConA, concanavalin A; IEF, isoelectric focusing; PAS, periodic acid-Schiff.
by counter-dialysis against a 10% (w/v) aqueous solution of PEG 6000; this constituted the water-soluble extract (WS). Before use in preparative IEF it was centrifuged at 100000 g for 1 h and the supernatant was dialysed against water overnight at 4 °C.

Preparative isoelectric focusing. Separations of WS extracts were done in a bed of Sephadex G-75 Superfine (Pharmacia) which had been pre-swollen, washed and dried with ethanol according to a published method (LKB, application note 198). A slurry was made by the addition of 5 g of dry Sephadex to 80 ml of a pre-mixed solution containing the required volume of extract (50-500 mg dry wt based on protein and sugar analyses) and 2-5% (w/v) of carrier ampholyte pH 3–10 or narrow-range pH 4–6.5 (Pharmalyte from Pharmacia). In later experiments Polybuffer 74 (Pharmacia) was substituted at a concentration of 10% (w/v) for the Pharmalyte (Pekkala-Flagan & Comings, 1982). The slurry was transferred to a special tray (110 x 245 x 5 mm) on a level table and spread with a straight-edged tool until evenly distributed. With very dilute solutions of antigen the volume of liquid was increased to 90 ml. In this case the slurry could be poured into the tray and it was set by sprinkling the surface (pepper-pot fashion) with additional, dry Sephadex until the gel remained stationary when the tray was raised to an angle of 45° from the horizontal. Electrode strips (artificial sponge from LKB) were soaked in 0.2 M-L-histidine (cathodic solution) and 0.04 M-DL-glutamic acid (anodic solution) and excess liquid was removed on blotting paper. They were placed in position by removing some of the gel slurry with a spatula from each end of the tray.

Electrophoresis at approximately 8 W was done over an effective separating distance of 23 cm for periods of 17-19 h in an LKB Multiphor apparatus with running water circulating through the cooling block. At the end of the separation period, two paper prints were taken for later visualization of the focused protein and glycoprotein constituents. The zones were collected by sectioning the gel bed with a fractionation grid resulting in 30 sections, each of which was removed with a spatula to a mini-column. Water was used for elution purposes and the volume of each fraction was adjusted to 4 ml. The $A_{280}$ and pH values were determined on each fraction. A portion of the eluted material was freeze-dried prior to protein and sugar analyses. The remainder was stored frozen at -20 °C.

Detection of separated zones. A special print technique was used (Radola, 1970) to visualize focused protein zones within the bed. Both prints were dried at 65 °C in a fan oven for approximately 30 min. One was washed in 10% (w/v) TCA, stained with 0.2% (w/v) Coomassie Brilliant Blue and destained in methanol/water/acetic acid. The other print was washed in absolute alcohol and stained by the periodic acid-Schiff (PAS) reagent (Hotchkiss, 1948).

Antigenic fractions of A. fumigatus. Solid ammonium sulphate was added to a WS preparation at increasing concentrations until saturation was reached (Hearn et al., 1980). One precipitate was used here, the 50-75% (NH$_4$)$_2$SO$_4$ (w/v) fraction (AS75) and the non-precipitable supernatant material (ASS).

In addition, a WS preparation was separated by affinity chromatography on ConA-Sepharose (Wilson & Hearn, 1983a). Two peaks were obtained, one of which eluted with the void volume and was protein-enriched (UBF fraction) and one which was elutable only on application of methyl α-D-mannoside and was carbohydrate-enriched (BF fraction).

Rabbit antisera. Antibodies to A. fumigatus were produced in New Zealand White rabbits. Antisera to crude wall material, a water-soluble extract (WS), UBF and BF fractions (material separated by affinity binding to ConA-Sepharose) were so previously described (Wilson & Hearn, 1982 and unpublished results). An antiserum (SBS) raised to a mixture of mycelial and culture filtrate antigens was a generous gift from Dr K. Holmberg of the State Bacteriology Laboratory, Stockholm, Sweden. An antiserum (X-9) raised to an alkaline extract of A. fumigatus mycelium (Lehmann & Reiss, 1978) was kindly provided by Dr E. Reiss, Center for Disease Control, Atlanta, Georgia, USA. Antisera raised to Candida albicans, Cryptococcus neoformans, Blastomyces dermatitidis, Histoplasma capsulatum, Coccidioides immitis and Paracoccidioides brasiliensis, and prepared as diagnostic reagents for use in double-diffusion tests, were generously donated by Dr L. Kaufman, Center for Disease Control, Atlanta, USA. Serum was collected from rabbits before injection of antigens for use as a negative control.

Human sera. Sera were obtained from patients suffering from aspergillosis and other pulmonary diseases, viz. asthma and cystic fibrosis. The sera had been either submitted to this laboratory or kindly donated by the Brompton Hospital, London, UK and Sully Hospital, Glamorgan, UK. Sera from patients with proven candidiasis, histoplasmosis, cryptococcosis or paracoccidioidomycosis were either specimens submitted to this laboratory, or were kindly provided by Dr L. Yarzabal, Instituto Nacional de Dermatologia, Caracas, Venezuela and Dr A. Restrepo, 'Pablo Tobon Uribe’, Medellin, Colombia. Sera which had no detectable antibodies for aspergillosis on counterimmunoelectrophoresis were used as negative controls on ELISA.

ELISA for detection of IgG antibodies. The method followed was that of Voller et al. (1979) using polystyrene microtitre plates (Dynatech, Plochingen, FRG; type M129A). All gel fractions were tested for their IgG-binding capacity at concentrations of 1 and 10 µg protein ml$^{-1}$. Most preparations gave maximal $A_{492}$ values when used at the 10 µg level. For comparative purposes, the water-soluble antigen from ruptured mycelium (WS) was included in the study. Its optimal concentration was batch-dependent and varied from 5 to 7.5 µg protein ml$^{-1}$. Sera were used at 1:100 dilution, except in cross-reactivity tests where a dilution of 1:200 was used. The enzyme conjugates were goat antihuman IgG and goat antirabbit IgG coupled to horseradish peroxidase (Miles Laboratories, Slough,
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UK) used at suitable dilutions. The substrate was o-phenylenediamine (BDH) and the A<sub>492</sub> was measured in a Titertek Multiskan spectrophotometer (Wilson & Hearn, 1983a).

Chemical analyses. The total protein content of unfractonated material and of each gel fraction was estimated by the dye-binding method of Read & Northcote (1981) with bovine serum albumin as a standard. Total neutral sugar was determined by the method of Dubois et al. (1956) with glucose as a standard. Coomassie Brilliant Blue G no. B-1131 was obtained from Sigma. All other reagents used were analytical grade.

RESULTS

In preliminary experiments, crude extracts of A. fumigatus were electrophoresed in a Sephadex layer which contained pH 3–10 ampholytes. A paper print, taken at the end of the electrophoresis period, and stained with Coomassie Blue, showed multiple protein zones focused between pH 4.0 and 5.2 with very little material visible outside a pH interval of 3.1–6.5. Subsequent focusing in a narrow-range ampholyte (pH 4–6.5) was used, and greater resolution of individual protein bands was achieved (Fig. 1a). Again the bands of greatest intensity were found in the pH range 3.5–5.2. Another paper print, treated with the PAS reagent, showed the bulk of the carbohydrate material to be associated with the major protein bands. The A<sub>280</sub> and pH were determined on each eluted fraction. Protein and carbohydrate analyses showed that the anodal material had a lower protein to carbohydrate ratio than did the cathodal material. The total recovery averaged 20% of the dry weight applied to a gel bed; some batches gave yields of 60%. The recovery of polysaccharide averaged 45–60% of that applied, while protein varied between 10 and 60%. The maximum load tested which gave adequate separation of individual bands was 420 mg dry weight; a load of 1 g proved excessive.

Reproducibility of the method. Repeated IEF experiments on samples of a single batch of water-soluble extract gave very reproducible separation profiles. Differences in distribution patterns became apparent with different batches of extract where additional components were sometimes found, and other components were either much reduced or absent. This phenomenon was especially marked with components of pI > 5.2.

Establishing a pH gradient. In later experiments Polybuffer 74, designed for use in chromatofocusing with ion-exchangers, was used to produce a pH gradient. Paper prints taken of components separated in the presence of Pharmalyte gave well-defined and more intensely-stained bands than those obtained with Polybuffer. However, while Pharmalyte gave satisfactory results, it was expensive to use; by substituting Polybuffer, the cost per experiment was reduced to approximately 10% of the original cost. Polybuffer gave a reproducible pH gradient while resolution and recovery appeared comparable to that obtained with Pharmalyte when tested in parallel experiments (cf. Pekkala-Flagan & Comings, 1982).

IEF fractions in an ELISA system. The antigenic activity of the isolated fractions was assessed in the ELISA system for IgG antibodies using a positive reference human serum pool and a negative human serum pool. A profile of antigen–antibody reactivity (measured by A<sub>492</sub>) is shown in Fig. 1(b). While all fractions bound antibody, three distinct peaks were found and fractions were pooled on this basis (i.e. peaks A, B and C). The position of these areas of reactivity varied somewhat from experiment to experiment. The most reactive peak was, in general, peak A (Table 1).

Comparison of IEF fractions with other A. fumigatus antigens. Antigens of A. fumigatus prepared by IEF were compared with those obtained by ammonium sulphate fractionation (fractions AS75 and ASS) and by lectin affinity chromatography (BF fraction). Dry weight recoveries of some fractions were low (Table 2). The IEF fractions and the BF fraction from affinity chromatography showed high immunological reactivity, as measured by ELISA. Mean values for the ammonium sulphate fractions were also high but considerable batch variation was found. Lowest batch variation was obtained with peak A.
1. Components of a water-soluble extract of *A. fumigatus* (batch 9) separated by IEF in Sephadex G-75 with carrier ampholyte of pH range 4.0–6.5. The photograph shows a paper print of the gel surface stained for protein with Coomassie Brilliant Blue G. (b) Reactivity of eluted fractions from IEF of *A. fumigatus* WS extract (batch 9) in an ELISA system. The $A_{492}$ of each fraction at 10 µg protein ml$^{-1}$ was measured (●). Fractions were pooled to give peaks A, B and C where distinct areas of reactivity were found. The pH gradient is shown (△).

![Figure 1.](image)

**Table 1. Comparative binding of IEF fractions and a water-soluble extract (WS) of *A. fumigatus* to clinically-defined sera in an ELISA system**

<table>
<thead>
<tr>
<th>Clinical diagnosis of individual patients</th>
<th>Binding of antigen preparation†:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peak A</td>
</tr>
<tr>
<td>Clinical asthma 1</td>
<td>1.180</td>
</tr>
<tr>
<td>Clinical asthma 2</td>
<td>0.243</td>
</tr>
<tr>
<td>Clinical asthma 3</td>
<td>1.275</td>
</tr>
<tr>
<td>Cystic fibrosis 4</td>
<td>0.731</td>
</tr>
<tr>
<td>Cystic fibrosis 5</td>
<td>0.819</td>
</tr>
<tr>
<td>Cystic fibrosis 6</td>
<td>0.979</td>
</tr>
<tr>
<td>Aspergilloma 7</td>
<td>1.155</td>
</tr>
<tr>
<td>Aspergilloma 8</td>
<td>0.145</td>
</tr>
<tr>
<td>Aspergillosis 9</td>
<td>1.499</td>
</tr>
<tr>
<td>Reference negative‡</td>
<td>0.081 ± 0.01</td>
</tr>
</tbody>
</table>

* No further clinical information available.
† A single batch of IEF fractions (batch 9) was used at their optimum concentration of 10 µg protein ml$^{-1}$; the results are shown as $A_{492}$ corrected for the negative control.
‡ Mean $A_{492}$ ± SD for five batches of peaks A, B and C tested against one negative human pooled serum sample; four batches of WS antigen (5–7.5 µg protein ml$^{-1}$) were tested against the same sera.

**Reactivity in ELISA of IEF fractions to rabbit antisera induced by a variety of *A. fumigatus* preparations.** Peaks A, B and C were tested for reactivity towards antisera raised in rabbits to total water-soluble material (WS) of *A. fumigatus* as well as antigenic fractions. The following end-points were obtained in titration: antiserum to WS, 1:25600 for all fractions; antiserum to a carbohydrate-rich fraction from affinity chromatography, BF, 1:25600 (peak A), 1:12800 (peak B) and 1:6400 (peak C); antiserum to wall, 1:6400 for all fractions; antiserum to mixed...
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Table 2. Comparison of IEF fractions and other antigenic preparations of A. fumigatus

<table>
<thead>
<tr>
<th>Antigen*</th>
<th>Percentage recovery†</th>
<th>Mean $A_{492}$ ± SD‡</th>
<th>Coefficient of variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak A</td>
<td>11.0</td>
<td>1.29 ± 0.16</td>
<td>12.0</td>
</tr>
<tr>
<td>Peak B</td>
<td>8.0</td>
<td>1.00 ± 0.29</td>
<td>29.0</td>
</tr>
<tr>
<td>Peak C</td>
<td>6.0</td>
<td>1.04 ± 0.28</td>
<td>27.0</td>
</tr>
<tr>
<td>AS75</td>
<td>6.0</td>
<td>0.80 ± 0.32</td>
<td>40.0</td>
</tr>
<tr>
<td>ASS</td>
<td>14.0</td>
<td>0.99 ± 0.45</td>
<td>46.0</td>
</tr>
<tr>
<td>BF</td>
<td>13.0</td>
<td>1.22 ± 0.28</td>
<td>23.0</td>
</tr>
<tr>
<td>WS</td>
<td>1.19 ± 0.33</td>
<td></td>
<td>28.0</td>
</tr>
</tbody>
</table>

* AS75, 50–75% (NH$_4$)$_2$SO$_4$ fraction; ASS, (NH$_4$)$_2$SO$_4$-soluble supernatant material; BF, fraction bound by ConA-Sepharose. The figures for these fractions have been reported elsewhere; UBF fraction was not included here as it was found to be unsuitable for ELISA (Wilson & Hearn, 1983a, b).

† Expressed as the sum of the protein and carbohydrate recovered for each sample; given as a percentage of the starting material (WS).

‡ Mean $A_{492}$ for five batches of peaks A, B and C, four batches of BF fraction and WS preparation and three batches of AS75 and ASS. One positive human pooled sample of serum was used.

Table 3. Comparative binding in ELISA of IEF fractions and a water-soluble mycelial extract (WS) to rabbit immunoglobulins induced by a variety of fungal species

<table>
<thead>
<tr>
<th>Infective agent*</th>
<th>Peak A</th>
<th>Peak B</th>
<th>Peak C</th>
<th>WS</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. fumigatus</td>
<td>1.494</td>
<td>1.414</td>
<td>1.570</td>
<td>1.345</td>
</tr>
<tr>
<td>C. albicans</td>
<td>1.046</td>
<td>0.212</td>
<td>0.094</td>
<td>0.707</td>
</tr>
<tr>
<td>B. dermatitidis</td>
<td>0.184</td>
<td>&lt;0</td>
<td>0.139</td>
<td>0.408</td>
</tr>
<tr>
<td>P. brasiliensis</td>
<td>0.098</td>
<td>&lt;0</td>
<td>0.052</td>
<td>0.183</td>
</tr>
</tbody>
</table>

* Rabbit antisera to these species were used here at a dilution of 1:200.

† A single batch of IEF fractions (batch 6) was used at their optimum concentration of 10 µg protein ml$^{-1}$; the results are shown as $A_{492}$ corrected for the negative control.

mycelial and culture filtrate antigens (SBS), 1:12800 (peak A), 1:6400 (peak B) and 1:3200 (peak C); an antiserum to an alkali-soluble extract (X-9) and one to a protein-rich fraction from affinity chromatography (UBF) showed minimal reactivity with all fractions.

Reactivity of IEF fractions to human sera in an ELISA system. Peaks A, B and C were tested against sera from individual patients diagnosed as suffering from aspergillosis or aspergillosis-related disease (Table 1; figures obtained with a water-soluble, antigenic extract are included for comparative purposes).

Cross-reactivity in ELISA of A. fumigatus IEF fractions to antibodies to species from other fungal genera. To determine the specificity for homologous antisera of the A. fumigatus fractions prepared by isoelectric focusing, peaks A, B and C were reacted with antisera raised in rabbits to species from several fungal genera (Table 3). For comparative purposes, reactivity of an unfractoniated mycelial extract (WS) towards these sera was tested simultaneously. Of the species studied, only Candida albicans showed a high level of cross-reactivity with peak A, while Blastomyces dermatitidis showed slight cross-reactivity with peaks A and C. The unfractoniated antigen, on the other hand, gave a relatively high level of cross-reactivity with both these fungal pathogens. No detectable cross-reactivity was seen when unfractoniated and partially-purified A. fumigatus antigens were tested with antisera to Cryptococcus neoformans, Histoplasma capsulatum or Coccidioides immitis.

The same antigens were then analysed for reactivity towards sera from patients with the corresponding mycotic infections. No $A_{492}$ value >0.14 was recorded with any of these antigens, and samples included ten cases of proven candidiasis and six cases of paracoccidioidomycosis. Unfortunately, no specimens of blastomycosis were available.
DISCUSSION

Preparative IEF yields antigenic fractions from a water-soluble extract of *A. fumigatus* which have some practical advantages over those prepared previously by either salt fractionation or lectin affinity chromatography (Hearn *et al.*, 1980; Wilson & Hearn, 1983a). Preparation time is reduced because fractions can be used, at least for ELISA, without either a concentration or a dialysis step. In addition, the combined recoveries of peaks A, B and C on a dry weight basis were somewhat higher by this method, and one fraction with a higher level of reproducibility of activity (defined by the coefficient of variation, towards a reference positive serum) was obtained. It has also been established that fractions may be stored either freeze-dried or frozen at -20 °C for several months without loss of immunological activity.

While the reactivity of fractions, especially peak A, is very reproducible on ELISA, the total recoveries of dry weight material vary enormously from batch to batch. This may be due to compositional differences in the starting material, in that some batches may contain a higher proportion of components whose pl values lie outside the pH interval 3.5-6.8. The possibility also exists that a number of proteins are rendered insoluble at their pl points and that this step is irreversible; the concentration of these components could also be batch-dependent.

Peak A material showed activity on ELISA comparable to that of an unfractionated extract of *A. fumigatus*. However, batch reproducibility was appreciably higher and binding of non-specific IgG by this partially-purified antigen was much lower. The IEF fractions showed comparable sensitivity in the detection of IgG antibodies in the two cases of proven aspergilloma investigated. With a number of clinical specimens, however, peak C was significantly less reactive than the other two fractions (Table 1). Five of six patients with clinically diagnosed asthma or cystic fibrosis showed high levels of specific IgG in ELISA tests. However, it is known that colonization by aspergillus may occur in these cases. Additional clinical information was not available to us, i.e. whether or not these patients showed overt symptoms of aspergillosis.

Peak A has similarities with pool 2, obtained by preparative IEF of culture filtrate antigens of *A. fumigatus* (Kurup *et al.*, 1983). The components have acidic pl values and are carbohydrate-enriched. In addition, peak A reacted strongly with an antiserum raised against an antigen (BF) separated through its affinity binding to ConA-Sepharose. Antigen IIb was subsequently separated from pool 2 on the basis of its ability to bind to ConA-Sepharose (Kurup *et al.*, 1983).

Cross-reactivity of peak A with an antiserum raised in rabbits to *C. albicans* was comparable to that seen when the same antigen was reacted with its homologous antiserum. This non-specific reaction was almost negligible with peaks B and C, which indicates significant differences in their composition. On the other hand, cross-reactivity of peak A was not detectable with serum from patients with candidiasis, shown to contain precipitating antibodies to *C. albicans* antigen on double-diffusion (unpublished results). This implies that there are differences between the antibody populations of experimental animals and patients, as far as detection in an ELISA test is concerned.

When tested for reactivity towards antisera raised to antigenic fractions of *A. fumigatus*, peaks A, B and C gave highest titres with an antiserum raised to a total WS preparation from homogenized mycelium. Intermediate values were given by antisera raised to the carbohydrate-rich BF fraction and crude wall material. Reactivities, especially of peak C, towards an antiserum to mixed mycelial and culture filtrate antigens (SBS) was low, while antisera to a protein-rich UBF fraction and an alkali-soluble extract (containing galactomannan and glucans; Dr E. Reiss, personal communication) gave titres of 1:100 or less. The results therefore indicate the presence of common components in the BF and IEF fractions, especially peak A. In general, peaks A and B showed similar reactivities with a given antiserum, while some differences were seen with peak C.

In the experiments described here, preparative IEF has resulted in the resolution of 10-14 readily identifiable protein zones in a single fractionation step. While this appears to give material suitable for use as diagnostic antigens in *in vitro* tests for aspergillosis, it must be emphasized that high molecular weight neutral polysaccharides will remain unfocused and distributed throughout the gel. This will give a degree of cross-reactivity among fractions. How-
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However, the process may be taken further, i.e. used in combination with other methods based on different properties of the molecules present, including size, and hence further purification of cellular components achieved. The technique shows potential as a general method for the separation of other fungal extracts.

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REFERENCES


